Investigations into senescence and oxidative metabolism in gentian and petunia flowers

A thesis

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Abstract

Using gentian and petunia as the experimental systems, potential alternative post-harvest treatments for cut flowers were explored in this project. Pulsing with GA_3 (1 to 100 μM) or sucrose (3%, w/v) solutions delayed the rate of senescence of flowers on cut gentian stems. The retardation of flower senescence by GA_3 in both single flower and half petal systems was accompanied by a delay in petal discoloration. The delay in ion leakage increase or fresh weight loss was observed following treatment with 5 or 10 μM GA_3 of the flowers at the unopen bud stage. Ultrastructural analysis showed that in the cells of the lower part of a petal around the vein region, appearance of senescence-associated features such as degradation of cell membranes, cytoplasm and organelles was faster in water control than in GA_3 treatment. In particular, degeneration of chloroplasts including thylakoids and chloroplast envelope was retarded in response to GA_3 treatment. In the cells of the top part of a petal, more carotenoids-containing chromoplasts were found after GA_3 application than in water control.

In petunia, treatment with 6% of ethanol or 0.3 mM of STS during the flower opening stage was effective to delay senescence of detached flowers. The longevity of isolated petunia petals treated with 6% ethanol was nearly twice as long as when they were held in water. Senescence-associated petal membrane damage, weight decline, ovary growth and decrease in protein and total RNA levels were counteracted in ethanol-treated petals. The accumulation of ROS, particularly superoxide and hydrogen peroxide, was also inhibited or delayed by ethanol application.

Anti-senescence mechanisms, particularly the changes of oxidative / antioxidant metabolism involved in petal senescence, were investigated. In gentian, activities of AP and SOD but not POD in the GA_3-treated petals were significantly higher than those of the control. In isolated petunia petals, the decreased trends of antioxidative SOD and AP

activities during senescence were apparently prevented in response to ethanol treatment although the levels of ascorbate and photo-protective carotenoids were not affected.

Furthermore, by optimizing a range of critical PCR parameters such as primer combinations, cDNA concentrations and annealing temperatures, a reliable protocol has been established for quantifying the expression level of Cu-Zn SOD gene in petunia petals using SYBR Green I based real-time RT-PCR. A 228 bp gene fragment of Cu-Zn SOD was isolated from petunia (var. 'hurrah') using RT-PCR. It was found that the mRNA level (relative to 18S rRNA level) of Cu-Zn SOD decreased significantly after 6 days in water. However, there was about a 55-fold increase in Cu-Zn mRNA level after 6 days of ethanol treatment when compared to water-treated petals. Similarly, down-regulation of the mRNA level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also observed during senescence of petunia petals. Increased vase life of petunia petals by ethanol treatment was correlated with promotion of GAPDH expression by a factor of about 16 on day 6. Taking together, the anti-senescence effects of GA_3 and ethanol are at least partially associated with an increased efficiency of petal system utilizing ROS since the selected antioxidants were significantly maintained when compared to the corresponding values for the control.

Keywords: Petunia; Gentian; Senescence; Flower; Ethanol; Gibberellic acid; Reactive oxygen species; Superoxide; Hydrogen peroxide; Antioxidant; Superoxide dismutase; Ascorbate peroxidase.

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Abbreviations

ANOVA	Analysis of variance
AP	Ascorbic acid peroxidase
ASC	Ascorbic acid
BA	6-benzyladenine
BLAST	Basic local alignment search tool
CAT	Catalase
cDNA	Complementary DNA
CLP	Chloroplast
CRP	Chromoplast
CW	Cell wall
CYCLO	Cycloheximide
DAS	σ-Dianisidine
DHA	Dehydroascorbate
DTT	Dithiothreitol
ER	Endoplasmic reticulum
FW	Fresh weight
GA_3	Gibberellic acid
GR	Glutathione reductase
GP	Glutathione peroxidase
GPD	Glucose-6-phosphate dehydrogenase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HEX	Hexokinase
8-HQS	8-Hydroxyquinoline sulphate
2 ip	N_6-[2-isopentenyl] adeosine
IS	Intercellular space
LOX	Lipoxygenase
M	Mitochondria

MDA	Malondialdehyde
MF	Membrane fragment
MW	Molecular weight
mM	Micromolar
mRNA	Message RNA
NADP	Nicotinamide adenine dinucleotide phosphate
NBT	Nitroblue tetrazolium
PCR	Polymerase chain reaction
PVP	Polyvinylpolypyrolidine
PAGE	Polyacrylamide gel electrophoresis
PG	Plastoglobuli
PGD	Phosphate glucose dehydrogenase
POD	Peroxidase
PUT	Putrescence
PLD	Pospholipase-D
PUFA	Polyunsaturated fatty acid
RT	Reverse transcription
SOD	Superoxide dismutase
SDS	Sodium dodecyl sulphate
SCF	SKP1, cullin/CDC53, F-box protein
SE	Standard error
STS	Silver thiosulfate
SAM	S-adenosyl methionine
SPM	Spermine
SPD	Spermidine
SFA	Saturated fatty acids
Tris	Tris(hydroxymethyl) aminomethane
TEM	Transmission electron microscope
UM	Micromillimole per litre
UPP	Ubiquitin-proteasome-pathway
UFA	Unsaturated fatty acids

CHAPTER ONE

INTRODUCTION

1.1 Plants used in this project

1.1.1 Gentian

Gentian, the common name of the genus *Gentiana*, includes about 400 species (Nelson, 1995) of annual or perennial (rarely biennial) flowering plants. They are classified in the division Magnoliophyta, class Magnoliopsida, order Gentianales, family Gentianaceae. Their distribution spans almost all continents throughout all climates. Although mostly in the alpine habitats of temperate regions of Asia, Europe and Americas, some species also occur in northwest Africa, eastern Australia and New Zealand (http://www.rci.rutgers.edu/struwe/gentet/gentian.htm/).

According to Pliny the Elder, the name of gentian derived from Gentius, an ancient King of Illyria (180-168 B.C.), who discovered the healing properties of gentian (http://en.wikipedia.org/wiki/gentian). It is said that gentian is probably the most well-known and studied pure bitter herb in the world (Hobbs, 1998). The importance of gentian is related to the medicinal values of various gentian species.

Furthermore, over the last decade, a range of gentian cultivars has been used as cut flower crops, the majority of which were sourced from Japan (Nelson, 1995). In New Zealand, gentiana has become a significant export cut flower crop (Eason et al., 2004). Their export remained relatively steady at 100,000 stems in the 2002 / 2003 season, their

main markets being North America and Europe (Anonymous, 2003). There will be a significant increase in gentian cut flower export from New Zealand since a new

variety, 'Ashiro gentian', was introduced from Japan to New Zealand. The Japanese co-operative targets the supply of two million stems from New Zealand growers and at least 50% of these for the Japanese market (Anonymous, 2002). In 2003, the Ashiro Gentian Growers' Group successfully obtained a Sustainable Farm Fund grant to develop and disseminate technology for growing 'Ashiro gentian' under New Zealand conditions (Anonymous, 2004a).

1.1.2 Petunia

The genus Petunia comprising about 30 (sub) species was established by Jussieu in 1803 and its main geographical distribution is from Argentina to Uruguay and in the southern part of Brazil as well as in the Andean foothills (Gerats and Vandenbussche, 2005). It is classified in the division Magnoliophyta, class Magnoliopsida, order Polemoniales, family Solanacea (Bowler, 1991) (www.cc.columbia.edu/cu/cup/). The common garden petunias, planted also in window boxes, are all considered hybrids of white-flowered and violet-flowered species from Argentina (Mittler et al., 2004) (http://www.petunia.cc/petunia.htm).

Petunia has been used to study diverse sets of interesting questions. It was mentioned as an outstanding model system in the foreword of the newsletter of the interim steering committee of the Plant Molecular Biology Association in June 1980 (Gerats and Vandenbussche, 2005). In this thesis research, Petunia 'Hurrah' plants were also used as they can be grown easily and produce a large quantity of flowers in the glasshouse throughout the year. Therefore, there was no problem with the availability of sufficient flowers at different developmental stages required for this study.

Plate 1. 1 Flowers of *Gentiana triflora*

Plate 1. 2 Petunia ‘Hurrah’ plants in a plant growth room

1.2 Cut flower industry in New Zealand

According to Horticulture Monitoring Report 2003, in New Zealand there were over 2,000 flower growers, approximately 400 as full time producers. The domestic market for cut flowers was estimated to be over $70 million annually, while the export of cut flowers had increased from $8 million in 1985 to $39 million in 2004 (Anonymous, 2004b). The total export value of flower and foliage sales in 2002 was $49.4 million, 5% lower than that of 2001 (Anonymous, 2002). The sale value was $40.4 million for the year ended March 2003, 20% lower than that of 2002. That for the year ended March 2006 was $38.5 million (Anonymous, 2006). There is a decreasing trend during the previous three years. That is possibly due to a number of issues: the poor seasonal weather and the weak market conditions, particularly the slowing down of the Japanese economy (Anonymous, 2003).

Even with an export decrease in recent years, a relatively mature cut flower industry is still around in New Zealand after a period of rapid worldwide expansion. It will continue to make a significant contribution to the local employment and economy in the future. However, in order to overcome the obstacles facing this market and maintain continuous growth in the cut flower industry, it is critical to identify and improve factors associated with increasing the quality and longevity of cut flowers.

In order to maintain high quality cut flowers with an optimal vase life, proper post-harvest care of cut flowers is necessary. The time of harvesting cut flowers should be taken into consideration. If it is too early, some flowers may not open and if it is too late vase life could be drastically reduced. Ethylene is another important consideration. It is commonly recognized as a factor affecting flower quality and limiting the marketing of many species of cut flowers. Cut flowers remain sensitive to damages and diseases, which will lead to ethylene production and premature senescence. Good ventilation and removal of dead / dying flowers or leaves may be essential to maintain a relatively ethylene-free environment. Finally, floral preservatives and other additives particularly

associated with counteracting ethylene production are necessary for post-harvest processing of ethylene-sensitive flowers such as gentian and petunia flowers.

1.3 Senescence and programmed cell death

Senescence is a complex, highly regulated physiological process in plant development. It is controlled at the genetic level. Coordinated macromolecule degradation, nutrient mobilization will eventually lead to the death of cell, tissue, organ and lastly the whole plant (Munne and Alegre, 2004; Parrott et al., 2007; Zimmermann and Zentgraf, 2004). In short, senescence can be described as the last developmental stage in the life cycle of a plant. This includes a series of endogenously controlled irreversible events that eventually lead to thorough cellular breakdown and death.

In plants, programmed cell death (PCD) is also an active process throughout development in response to both abiotic and biotic stresses. It is a programme leading to death and is simply referred to as the death of cells (Slooten et al., 2001; Swidzinski et al., 2004). The mechanisms of PCD in plants bear a certain relation to those of apoptosis, and some processes, such as nucleic acid degradation, are superficially similar to aspects of the senescence syndrome (Thomas et al., 2003). It has been reported that nuclear DNA degradation is an early regulatory event rather than a result of massive cell death in the final stage of senescence. This may be a key difference between PCD and senescence (Hoeberichts et al., 2005).

In flowers, petal senescence is characterised by the visible signs of petal wilting and inrolling. It is an important determinant of the quality of cut flowers (Yamada et al., 2003). Petal senescence is a form of PCD with features of animal apoptosis (Hoeberichts et al., 2005). A number of characteristics associated with PCD such as chromatin condensation, a decrease in nuclear diameter and DNA fragmentation, also occur during cell death leading to petal senescence (Arora and Singh, 2006; Yamada et al., 2003). However, abscission of turgid petals at the end of floral life is not always preceded by a number of PCD indicators (Yamada et al., 2007a).

In addition, the term senescence is often confused with aging, which refers to the process of accruing maturity with the passage of time and induces senescence (Munne and Alegre, 2004; Thimann, 1980). It encompasses a wide array of passive degenerative processes that occur over time without referring to death as a consequence. It is driven primarily by external factors, for example the changes in seasons (Rodriguez et al.1990; Torelli, 2004).

1.4 Senescence: structural and functional changes at the cellular and biochemical level

Senescence is an endogenously controlled degenerative process leading to cell, tissue, organ and plant death. Multiple catabolic pathways involved are responsible for the degradation of cell constituents, such as proteins, polysaccharides, lipids, nucleic acids, and pigments (Narumi et al., 2006). A variety of hydrolytic enzymes play important roles in the catabolism of macromolecules. This in turn sets in motion disassembly of membrane molecular matrices, leading to loss of cell function. Ultimately, complete breakdown of cellular ultrastructure will result (Hopkins et al., 2007).

1.4.1 DNA fragmentation and nuclear degradation

It has been reported that loss of nucleic acids including DNA and RNA is associated with plant senescence (Aleksandrushkina et al., 2008; Crafts-Brandner et al., 1998; Rubinstein, 2000). Nucleases are thought to play a role in the senescence-induced massive degradation of nucleic acid content. The extent of nucleic acid degradation is closely related to the elevation of total nuclease activity associated with age (Aleksandrushkina et al., 2008).

It has been found that activities of various nucleases such as RNase and DNase increase during senescence (Canetti et al., 2002; Lers et al., 2006; Panavas et al., 2000). The bifunctional nucleases which are able to degrade both RNA and DNA have been

identified. Their activities and the levels of mRNA encoding them increase during plant senescence (Canetti et al., 2002; Perez-Amador et al., 2000).

Modifications of nucleic acids during senescence have frequently been reported. In the senescing cork tissue, accumulation of mRNA and DNA point modifications has been found but not in the young tissue of *Quercus suber* (cork-oak tree) (Pla et al., 2000). DNA fragmentation is also associated with plant senescence (Eason et al., 2002c; Panavas et al., 2000). A large increase in the number of DNA masses (indicating chromatin fragmentation) and a decrease in the diameter of nuclei and DNA masses (due to chromatin condensation) have been observed during programmed cell death (PCD) of petals (Yamada et al., 2006).

1.4.2 Protein degradation

In general, proteins are the key molecules that play important roles in various structural and functional aspects of plants. An important event during senescence in plants is protein degradation and remobilization. This is mediated in many species through protein ubiquitination and the action of specific proteases (Pak and van Doorn, 2005; Wagstaff et al., 2002).

Senescence of petals is a highly regulated developmental process which requires active gene expression and protein synthesis. It has been found that the protein levels of soluble, microsomal- and plastid-enriched fractions in the petals of daylily (*Hemerocallis hybrid* cv. Stella d'Oro) decrease, whereas the activities of at least three classes of proteinases increase during senescence (Stephenson and Rubinstein, 1998). Also some free amino acids are known to increase significantly after the onset of senescence (Wen et al., 1996).

Ubiquitin is involved in the degradation of many petal proteins during floral development and senescence. Some of ubiquitinated proteins increase in intensity as the flowers senesced. During senescence, this selective degradation is occurring against a background of net proteolysis (Courtney et al., 1994).

1.4.3 Lipid degradation

The decrease in levels of various lipids is associated with plant senescence. It has been found that phospholipids and galactolipids are extensively degraded during flower senescence and the ratio of saturated / unsaturated fatty acids increases (Leverentz et al., 2002). The decline in the content of membrane components such as phospholipids is also a key event in flower senescence (Torre et al., 1999). During petunia (*Petunia hyhrida*) flower senescence, there is a senescence-related increase in the content of diacylglycerol, one of the products of phospholipids metabolism, in plasma membranes (Borochov et al., 1997). Microsomal membranes from the petals of senescing carnation (*Dianthus caryophyllus L.*) flowers contain phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylinositol. These phospholipid classes decline essentially together during natural senescence of the flower (Brown et al., 1991).

1.4.4 Pigment and chloroplasy degradation

Chlorophyll is the most abundant photosynthetic pigment in higher plants. During senescence, chlorophyll breakdown is a prerequisite for the remobilization of nitrogen from chlorophyll-binding proteins to proceed during senescence (Hortensteiner, 2006). It has been found that most chlorophyll species breakdown during senescence (Louda et al., 2002; Spundova et al., 2003). However, during senescence carotenoids may show different behaviours. There could be a decrease, increase or no change depending on pigment species (Suzuki and Shioi, 2004). Induction of carotenoid synthesis during senescence provides protection for plastoglobuli and light-sensitive constituents of plant tissues from irradiation (Merzlyak and Solovchenko, 2002). Carotenoids remain relatively more stable than chlorophylls (Biswal, 1995).

With regard to the chloroplast ultrastructure a higher level of thylakoid disorganisation (especially of granal membranes) is observed during senescence (Spundova et al., 2003).

Various external factors can induce oxidative stress and senescence in chloroplasts. This often results in structural damages and dysfunctions such as swelling of thylakoids, disruption of chloroplast membranes, an increase in the number and size of plastoglobuli, photo-destruction of pigments and inhibition of photosynthesis (Kolodziejek et al., 2003; Mostowska, 1999). Chloroplasts are prone to oxidative damage (Allen et al., 1997). However, degradation of chloroplast structure is not always correlated with a decrease in photosynthetic pigment content (Kolodziejek et al., 2003).

The trend of a decrease in photosynthetic rate and pigment level is generally accepted to be due to the involvement of oxygen radicals and singlet oxygen (Dertinger et al., 2003; Prochazkova et al., 2001). It is reported that as plants age effects of oxidative stress accumulate progressively in chloroplasts and the contribution of oxidative stress to aging increases (Munne and Alegre, 2002). An enhanced lipid peroxidation and degradation of antioxidants in chloroplasts such as beta-carotene and alpha-tocopherol are also observed during senescence (Munne et al., 2001).

Anthocyanins found in vacuoles can be induced during leaf senescence (Garcia et al., 2003; Lee, 2002). In flowers, degradation of anthocyanins during senescence is possibly related to oxidative process. It has been reported that a significant increase in peroxidase activity is correlated with the rate of anthocyanin degradation. Reducing agents such as glutathione can inhibit the degradation of anthocyanins (Vaknin et al., 2005).

1.4.5 Membrane degradation

Cellular membranes are selective, dynamic barriers that play an essential role in regulating biochemical and physiological events in cells. Structural integrity of membranes is necessary for critical membrane functions such as maintaining the cell's osmotic balance in petals. The rupture of cellular membrane is likely to be deleterious to plant function since regulation of metabolite and signal exchange between neighboring cells will be lost. Membrane damage may occur early or later in the process of senescence. It is said that membrane may be the first target for degradation in the French

bean nodule senescence process (Puppo et al., 1991). However, membrane breakdown in lupin was only observed at a very advanced stage of senescence (Hernandez et al., 2002). Senescence of petals is also accompanied by numerous well-defined compositional, structural and functional changes in their cellular membranes (Beja-Tal and Borochov, 1994). The appearance of visual signs of petal senescence in cut flowers also are related to the changes in membrane permeability (Celikel and Vandoorn, 1995; Kondrat'eva and Belynskaya, 1995). A marked deterioration of the plasma membrane and loss of water, which is associated with lipid peroxidation, is associated with the senescence of chrysanthemum petals (Bartoli et al., 1997b; Bartoli et al., 1995). With loss of the integrity of cellular membrane structure in cells undergoing senescence, hydrolytic enzymes being normally compartmentalized in these cells are released and could cause massive breakdown of various cellular components.

1.4.6 Mitochondria degradation

Mitochondria have long been suspected to be involved in the biological aging process. They posses their own genetic material (mtDNA), but only with a limited DNA repair system. This makes them one of the prime targets for reactive oxygen species (Bohr and Anson, 1999; Kowald, 2001). Dysfunctional mitochondria with the inadequacies in the protection from reactive oxygen species and the oxidative damage in mitochondria may play a key role in oxidative stress and senescence (Anson and Bohr, 2000). Increased production of reactive oxygen species (ROS) by mitochondria is involved in oxidative damage to the organelle and in committing cells to senescence (Basu et al., 2001). However, it is reported that plant mitochondria may remain functional until the final stages (Quirino et al., 2000). No significant disintegration was observed in mitochondria during senescence of the corolla of the four-o'clock flower (*Mirabilis jalapa* L.) (Li et al., 1994). However, the dilation of the outer mitochondria membrane is observed in carnation petal cells during the pre-climacteric stage (Smith et al., 1992). The damage to internal mitochondrial membrane has been detected in mesophyll cells during senescence (Kolodziejek et al., 2003). The relatively stable mitochondria structure might indicate a

continued need for energy by the various active processes of senescence, for example, metabolite reallocation.

1.5 Senescence: changes in oxidative metabolism

1.5.1 Generation of reactive oxygen species in plants

A free radical is an atom or molecule with an unpaired electron, which usually seeks to accept (or donate) an electron from an adjacent molecule to become paired and stable (Nooden and Leopold., 1988). In aerobic organisms, an inevitable result from plasma membrane-linked electron transport is the leaking of electrons onto molecular oxygen (Fridovich, 1995). This sequential reduction of molecular oxygen with varying number of electrons results in the formation of reactive oxygen species (ROS).

With regard to the locations, ROS can be generated in organelles with a highly oxidizing metabolic activity or with an intense rate of electron flow, such as chloroplasts, mitochondria and microbodies during normal cellular metabolic activity (Mittler et al., 2004). Both the cytoplasm and the endoplasmic reticulum have also been reported to produce ROS in detoxifying reactions catalyzed by cytochromes (Urban et al., 1997). Moreover, pH-dependent cell wall peroxidase, oxalate oxidases and amine oxidases can generate ROS at the apoplast (Smirnoff, 2005; Vranova et al., 2002). In peroxisomes, hydrogen peroxide can be produced by the enzymatic activity of glycolate oxidase, and in microbodies as a side-product of fatty acid oxidation during lipid catabolism (Mittler et al., 2004). Plasma membrane NADPH-dependent oxidases contain a multimeric flavocytochrome that forms an electron transport chain capable of reducing O_2 to O_2^-. They have been thought to play a key role in ROS signaling (Mittler et al., 2004).

ROS are generally classified into two groups: (a) non-free-radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen radicals ($^1O_2$), and (b) free radicals such as superoxide anion radical and hydroxyl radicals ($OH^{\cdot}$) (Gulcin et al., 2005). The interaction of reactive oxygen species derived from oxygen is shown in *Figure 1.1*.

Singlet oxygen ($^1O_2$) is generated by direct donation of excitation energy from chlorophyll to oxygen in photosynthetic electron transport. It is also highly reactive with organic molecules due to an electron elevated to a higher energy orbital (Smirnoff, 2005). Superoxide anion is generated by single electron reduction in the electron transport system of mitochondria and/or chloroplasts. $O^{\cdot-}_2$ can exist in equilibrium with its conjugate acid, hydroperoxyl radical ($HO_2^{\cdot}$). Two superoxide radicals may react spontaneously or by the action of SOD to form hydrogen peroxide and oxygen (Kanazawa et al., 2000). Moreover, hydrogen peroxide can react with transition metals such as iron or copper to form the highly toxic $HO^{\cdot}$ radical via the Fenton reaction or Haber-Weiss reaction (Bowler, 1991; Halliwell and Gutteridge, 1989).

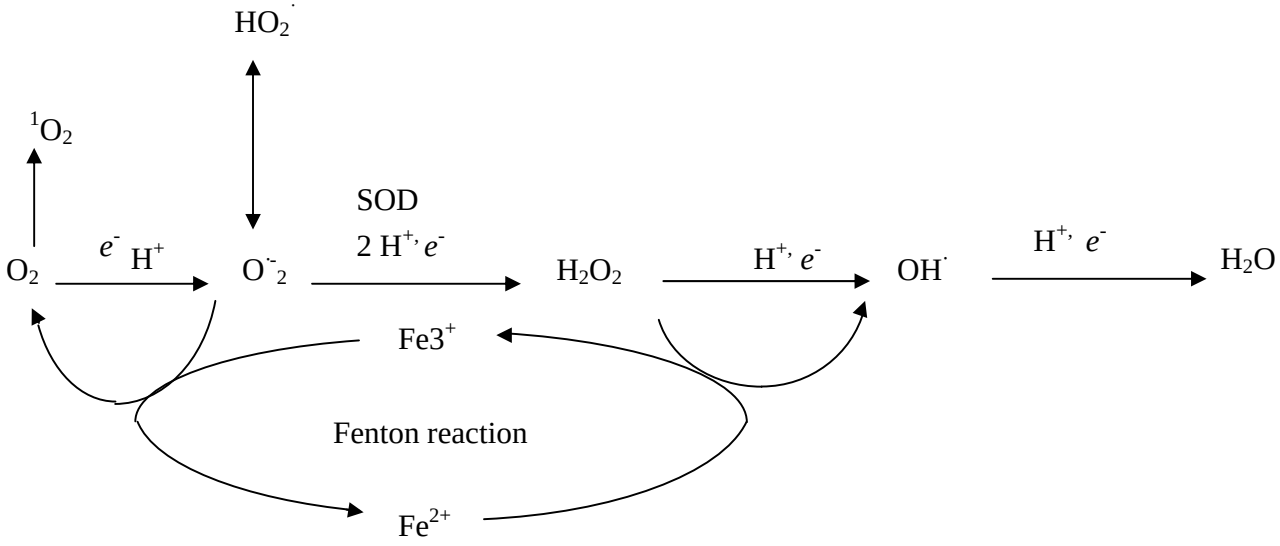


Figure 1. 1 Interaction of reactive oxygen species derived from oxygen.
SOD, superoxide dismutase; $^1O_2$, singlet oxygen radicals; $O^{\cdot-}_2$, superoxide anion; $HO_2^{\cdot}$, hydroperoxide radical; $OH^{\cdot}$, hydroxyl radical (Vranova et al., 2002).

1.5.2 Antioxidant defence in plants

Oxidative metabolism has reactive oxygen species (ROS) as unavoidable by-products. However, plants possess several mechanisms to protect cells against the toxic effects of ROS (Tanyolac et al., 2007). One is to use enzymatic antioxidant system (*Figure 1.5*), and another one is to use a system of non-enzymatic antioxidant substances (Halliwell and Gutteridge, 1989; Podsedek, 2007). These two groups usually act in tandem (Foyer et al., 1994).

Major ROS-scavenging enzymes of plants include SODs, a series of H_2O_2-scavenging enzymes such as ascorbate peroxidase, catalase, glutathione peroxidase and peroxiredoxin (Mittler et al., 2004). Together with antioxidant substrates ascorbic acid and glutathione, these enzymes play a crucial role in determining a steady-state level of superoxide and hydrogen peroxide. SOD plays a central role in the enzymatic ROS-scavenging pathways (Ma and Zhu, 2003). As the first enzyme in the detoxifying process SOD catalyses the transformation of $O_2^{\bullet -}$ to O_2 and H_2O_2 (Guo et al., 2006). In ascorbate-glutathione cycle, the resulting hydrogen peroxide is converted to H_2O by ascorbate-dependent peroxidase (APX, EC 1.11.1.11) (Kanazawa et al., 2000; Zimmermann et al., 2006). The oxidized ascorbate is re-reduced by NAD(P)H or GSH in the reaction catalyzed respectively by dehydroascorbate reductase (DHAR, EC 1.6.5.4) and monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) (Kanazawa et al., 2000). Glutathione peroxidase is responsible for removal of H_2O_2 and organic hydroperoxides produced by lipid peroxidation. It oxidizes reduced glutathione (GSH) to oxidized glutathione (GSSG) which is finally reduced again by NADPH in a reaction catalyzed by glutathione reductase (GR EC 1.6.4.2) (Cheeseman and Slater, 1993).

Scavenging H_2O_2 can also be mediated in plants by non-specific (guaiacol-dependent) peroxidase (GPX, EC 1.11.1.7). It is involved in various biosynthetic pathways including lignin synthesis, which also uses H_2O_2 (Moreno et al., 2001; Paliyath and Pinhero, 2000;

Srivalli et al., 2003). Catalase (CAT, EC 1.11.1.6) is also involved in the conversion of hydrogen peroxide (Casp et al., 2002; Kukavica and Jovanovic, 2004).

Antioxidant substances can be lipophilic or hydrophilic in either the lipid or aqueous portion of cells. Ascorbic acid (a water-soluble antioxidant) can directly react with superoxide and scavenging singlet oxygen (Bashor and Dalton, 1999; Casano et al., 1997). It participates in the removal of hydrogen peroxide as a substrate of ascorbate peroxidase (Casano et al., 1997). Carotenoids are water-insoluble and have a remarkable anti-oxidant potential as well (Rocha-Ribeiro et al., 2007). They are involved in preventing the formation of singlet-oxygen by quenching triplet-state chlorophyll. They can also scavenge any singlet oxygen as well as hydroxyl radical and peroxyl radicals generated in the process of lipid peroxidation (Biswal et al., 1994; Tapiero, 2004; Young, 1991). Another lipid soluble antioxidant, α-tocopherol, is located in membranes and is believed to be the major defense system against lipid peroxidation since it can react with lipid peroxyl and alkoxyl radicals (Dhindsa et al., 1982; Fryer, 1992; Kunert and Ederer, 1985; Munne-Bosch et al., 2007). Anthocyanins from various vegetables and fruits have antioxidant activities (Han et al., 2006b). They could be beneficial in scavenging free radicals and reducing lipid peroxidation (Duan et al., 2007). The function of anthocyanins in vegetative tissues is a contentious issue. They may act as photo-protective light screens due to their ability to reduce excitation pressure (Peng et al., 2006; Steyn et al., 2002). It was reported that the accumulation of anthocyanins was increased in *Arabidopsis* following treatment with a radical-generating reagent (Nagata et al., 2003; Steyn et al., 2002).

1.5.3 Senescence and oxidative stress

ROS can react with all major biological macromolecules such as carbohydrates, nucleic acids, lipids, and proteins. The term of oxidative stress is described as any condition in which cellular redox homeostasis is disrupted, with more peroxidative than antioxidative reactions (Bartosz, 1997; Yu, 1996). Although plants are equipped with several lines of defence against oxidative stress, these defence mechanisms are still not 100% effective. It

has been reported that both biotic and abiotic stresses are associated with senescence (Ohme et al., 2000; Pastori and Trippi, 1993; Prochazkova et al., 2001; Yan et al., 2000). The involvement of oxidative stress is considered to be one of the major causal factors of senescence (Dubey et al., 1996; Hernandez et al., 2002; Wiersma et al., 2004).

The most reactive forms of ROS known are $OH^{\cdot}$ and $^{1}O2$, derived from superoxide and hydrogen peroxide (described in detail in section 1.7.1). One of their deleterious reactions is the peroxidation of the cell membrane's unsaturated lipids to form unstable peroxides, which then decompose to the highly reactive free radicals. They can continue to propagate the vicious lipid peroxidation chain reaction and eventually threaten cellular integrity and function. The hydroperoxide dependent-lipoxygenase (LOX) pathway has been known to be involved in the initiation of lipid peroxidation (Yu et al., 2006). In postharvest biology, increased LOX-dependent membrane lipid peroxidation is regarded as a major characteristic during senescence (Bartoli et al., 1995; Srivalli et al., 2003). Malondialdehyde (MDA) is an end product of lipid peroxidation caused by reactive oxygen species and one of the most frequently used indicators of lipid peroxidation. It has been reported that the membrane permeability and the level of MDA content increase during petal senescence (Sun et al., 2004). Cut carnation senescence is also associated with the increase in both the efflux of electrolytes (membrane damage index) and 2', 7'-dichlorofluorescein oxidation rate (oxidative stress index) (Bartoli et al., 1996).

However, solute leakage of iris tepal is not due to the peroxidation of membrane lipids since the rate of ethane production, an indicator of lipid peroxidation, was very low and remained unaltered during senescence (Celikel and Van, 1995). Accumulation of lipid hydroperoxides were also not associated with the loss of membrane function in *Alstroemeria peruviana* flowers (Leverentz et al., 2002).

Senescence is highly complex, involving multiple mechanisms at different levels (von et al., 2001). Lipid peroxidation is one of the most important mechanisms contributing to plant senescence. Except for malondialdehyde, the level of carbonyl group resulting from protein oxidation is also used as an oxidative stress marker to reflect the degree of

cellular oxidative damage, and an increased level in protein carbonyl groups has been reported to be associated with senescence (Hernandez et al., 2002; Todorova et al., 2002). Hydroxyl radical can also oxidize amino acid residues of protein, which in turn may lead to enzyme inactivation, protein dysfunction and membrane disruption. The senescence of chrysanthemum petals was accompanied by an increase in the content of oxidized proteins which were recognized and degraded by the proteasome, a multicatalytic proteinase complex (Bartoli et al., 1997a; Merker and Grune, 2000). Ubiquitin-proteosome system played a causal role in petal senescence, and the inhibitors of proteosome activity delayed ion leakage (Rubinstein, 2000; Stephenson and Rubinstein, 1998). Recent evidence also suggests that oxidative DNA damage is known to play a very important role during senescence (Chen et al., 1995; Sohal, 2002; von et al., 2001).

1.5.4 Senescence and antioxidants

Since ROS levels and their damage products in plants are known to increase during senescence, it is possible that these changes are due to a decline in the levels of certain antioxidants (Ye et al., 2000).

The decrease in levels of a lot of antioxidant substrates is generally associated with plant senescence. It has been found that the levels of water soluble ascorbic acid and glutathione decrease in plant tissue during senescence (Bartoli et al., 1997a; Serrano et al., 2006). A reduction in the antioxidant substrate carotenoid has also been reported to be closely related to senescence (Feng et al., 2003; Woo et al., 2004). Polyunsaturated fatty acids, which are highly sensitive to ROS, were found to be decreased during aging (Anantharaju et al., 2002; Barata et al., 2005). In *Alstroemeria peruviana*, the ratio of saturated / unsaturated fatty acids in both sepals and petals increased during senescence (Leverentz et al., 2002).

With regard to antioxidative enzymes, a previous study has shown that there was an increase in ion leakage before daylily petals showed visible signs of degradation. The membrane changes leading to cell death were induced partially by the increased activity

of lipoxygenase and level of ROS. These were related to the reduced effectiveness of certain protective enzymes such as catalase and ascorbate peroxidase (Panavas and Rubinstein, 1998a).

In some cases, an initial increase in antioxidant substrates is possible. For example, the concentration of alpha-tocopherol was increased just at the onset of aging, and decreased as aging progressed in *Chrysanthemum morifolium* RAM flowers (Bartoli et al., 1995; Dertinger et al., 2003). The level of reduced thiols was augmented and the oxidized / reduced thiol ratio was enhanced during lupin (*Lupinus albus* L. cv. Multolupa) nodule senescence as well (Hernandez et al., 2002).

Similarly, an early increase in the activities of antioxidant enzymes such as SOD, catalase, peroxidase, and AP has been shown to occur during the initial stages of flower senescence although a decrease trend occurs at a later stage (Bartoli et al., 1995; Sun et al., 2004). In daylily, the activities of superoxide dismutase and peroxidase have been reported to increase during flower senescence (Panavas and Rubinstein, 1998a). During the storage of broccoli flower buds, there is also a correlation between the senescence indices and increasing antioxidant enzyme activities (Woo et al., 2004). When polyethylene foil was used for packaging of broccoli heads during storage, the activities of SOD, CAT, POD decreased while chlorophyll decomposition in flower buds was slowed down (Starzynska et al., 2003).

Sometimes antioxidant defense enzymes are not responsive to early senescence-related oxidative stress. It has been reported that the early processes of senescence was not related to the diminished free radical scavenging in iris tepal by SOD, APX since low SOD and APX activities were found until the display of visible senescence symptoms (Bailly et al., 2001). Degradation of chlorophyll (chl) during natural senescence of a leaf has been reported not to be accompanied by a reduced level of ascorbate (Kukavica and Jovanovic, 2004).

In plants, antioxidant defences are triggered by coordinated mechanisms to control oxidative damage during senescence. The transcription of the GST1 gene encoding a subunit of glutathione-S-transferase has been found to be regulated by ethylene during carnation petal senescence (Itzhaki et al., 1994). The aging process can be counteracted by an enhancement of the antioxidant defense system to attenuate free radical-induced damage (Satoh et al., 2004). Although antioxidant defence is very important, it is just one of the many mechanisms involved in the process of senescence. It is possible that the levels of antioxidants in plants may increase, decrease, or remain invariable during plant senescence.

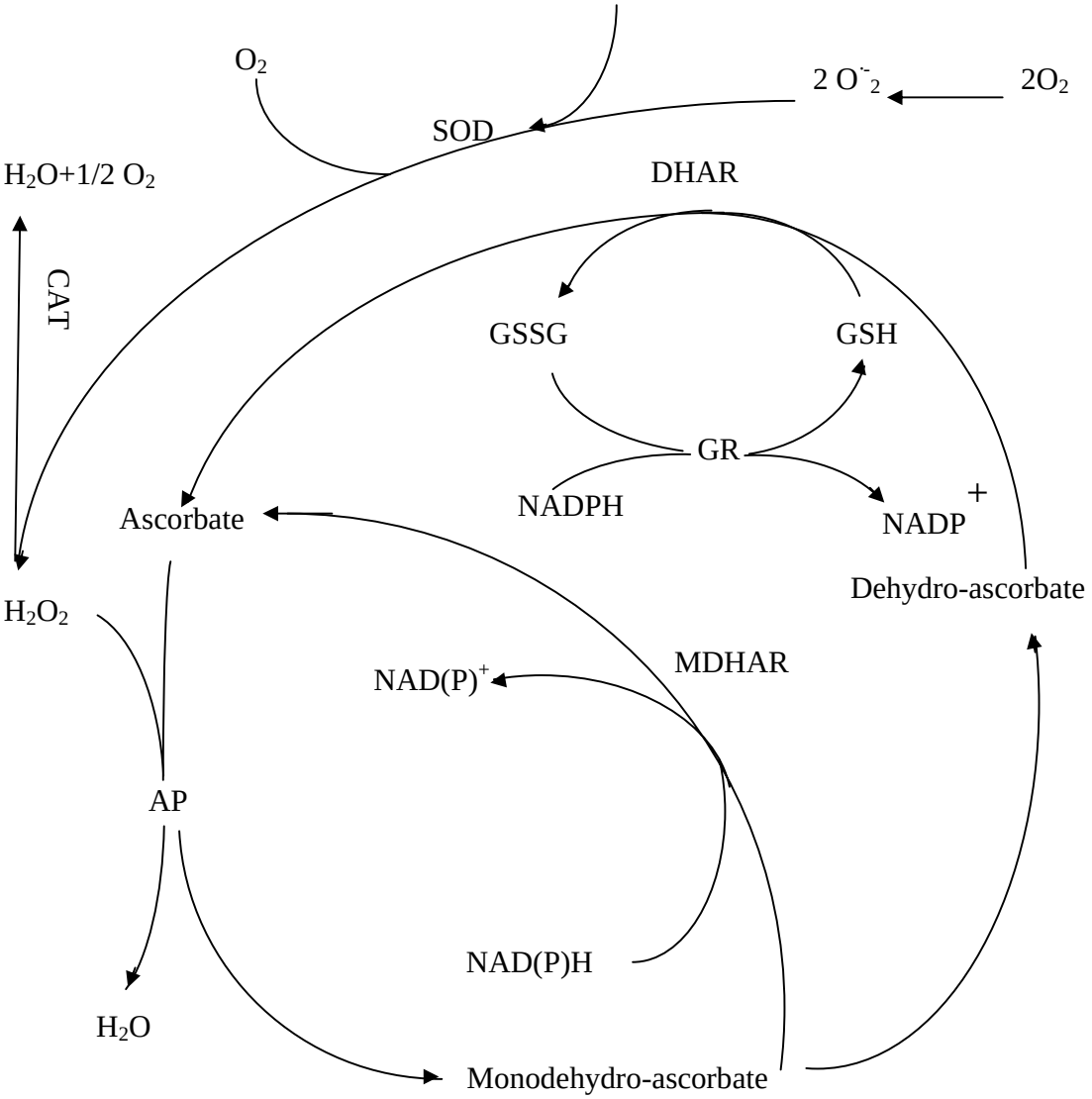


Figure 1. 2 The ascorbate-glutathione redox cycle

SOD, superoxide dismutase; AP, ascorbate peroxidase; CAT, catalase; MDHAR, monodehydro-ascorbate reductase; DHAR, dehydro-ascorbate reductase; GR, glutathione reductase (Buchanan et al., 2000).

1.6 Gene expression and regulation associated with senescence

1.6.1 Senescence-associated genes

According to Nooden (1997), genetically-based programs underpin senescence and cell death. A series of unique genes such as photosynthesis-related genes might have been inactivated or down-regulated during the final developmental and senescence period. However, the term of 'senescence-associated genes' is used to describe genes that are expressed more actively during senescence (Falk et al., 2002; Panavas et al., 1999; Thomas et al., 2003; Woo et al., 2004; Yamada et al., 2007b). Expression of a number of genes involved in the degradation and remobilization of macromolecules such as proteins, lipids, nucleic acids and cell wall compounds is known to be upregulated during petal senescence (van Doorn et al., 2003; Xu et al., 2006). The expression of genes encoding cysteine proteinases, nuclease (DcNUC1) and pectinacetylesterase involved in cell-wall degradation is increased during petal senescence (Eason et al., 2002c; Narumi et al., 2006; Panavas et al., 1999; Yamada et al., 2007b). GenBank database homology searches indicate that one cDNA, whose level increases during daylily (*Hemerocallis hybrid*) petal senescence, is most similar at the amino acid level to an in-chain fatty acid hydroxylase (Panavas et al., 1999). In rose, a cDNA for senescence-inducible gene exhibits a significant sequence homology to that of *delta-9-desaturases*, which play an important role in the degradation of saturated fatty acids of membrane lipids (Mizutani et al., 1995).

Furthermore, the-upregulated senescence-associated genes identified to date encode a variety of proteins including transcription regulators and signal-transduction proteins. WRKY proteins comprise a family of plant specific zinc-finger-type transcription factors that seem to be involved in the regulation of gene expression during leaf senescence (Miao et al., 2004). A number of other up-regulated regulatory genes has been identified during leaf or flower senescence (Gregersen and Holm, 2007; van Doorn et al., 2003). A gene encoding a RING zinc finger ankyrin repeat protein (MjXB3), a putative E3 ubiquitin ligase, is highly expressed in petals of senescing four o'clock (*Mirabilis jalapa*)

flowers (Xu et al., 2007). The expression of senescence-related transcription factor PeWRKY6-1 is also induced in the leaves of Pelargonium cuttings (Rosenvasser et al., 2006).

Senescence-associated signal transduction may involve G-proteins, calcium activity changes and the regulation of protein phosphorylation and dephosphorylation (Rubinstein, 2000). It has been reported that the transcripts encoding senescence-associated receptor-like kinase (SARK) in bean (*Phascolus vulgaris*) leaf, a leucine-rich repeat receptor protein kinase and a 14-3-3 protein (a protein kinase) in morning glory (Ipomoea nil) flowers increase during senescence (Hajouj et al., 2000; Yamada et al., 2007b).

Moreover, some of these senescence-associated genes have been implicated in the phytohormone pathways. The well known examples are genes for the ethylene biosynthetic enzymes ACC oxidase and ACC synthase, and those for ethylene receptor. They increase in abundance during flower senescence (Jones and Woodson, 1999; Muller et al., 2000; Payton et al., 1996). In addition, numerous genes, whose functions are not yet known, have been reported to be upregulated during flower senescence (Hoeberichts et al., 2007; Hunter et al., 2002; Panavas et al., 1999).

1.6.2 The technical approach for the study of gene expression used in this project

Changes in the expression of genes coding for antioxidant enzymes may be involved in the protection of plants against oxidative stress. So far, Northern blotting, a time-consuming technique and requiring a large quantity of RNA, is the common methodology to analyse the level of gene expression (Dean et al., 2002). RT-PCR is a technique which begins with converting RNA back to complementary DNA, and then amplifying the DNA sequence by using specific primers matching the target gene (Baek et al., 2004). Currently, reverse transcription (RT)-PCR-based assays are commonly used for characterizing or confirming gene expression pattern and comparing gene levels in

various sources (Mocellin et al., 2003). These are particularly useful in situations where amounts of RNA are insufficient for Northern blot analysis (Woo et al., 2004).

Quantitative real-time RT-PCR, based on the use of fluorescently labeled sequence-specific hybridization probes or DNA binding dye, is used when accurate quantification of the levels of a specific RNA or DNA target is required (Deprez et al., 2002). Both rely on measuring the intensity of a fluorescent signal that is proportional to the amount of DNA during early PCR amplification (Wittwer et al., 1997).

1.6.2.1 Advantages of using real-time RT-PCR to detect gene expression

1.6.2.1.1 Sensitivity

Firstly, real-time PCR technology provides a higher technical sensitivity for the detection of nucleic acid due to a combination of the sensitivity of conventional PCR with the generation of a specific signal by means of a probe or a dye (e.g. SYBR Green I), capable of detecting fewer than 5 copies of a DNA molecule (Klein, 2002). It does not require agarose gel electrophoresis, which has a limited dynamic range for analysis of PCR products. Also SYBR Green I is more sensitive than ethidium bromide for DNA detection. It is reported that the analytical sensitivity of real-time RT-PCR assay is 100 times higher than that of the conventional RT-PCR (Munir and Kibenge, 2004).

1.6.2.1.2 Rapidity

In addition to its higher sensitivity, the SYBR Green I-based real-time PCR assay is also more rapid in providing reliable data than the conventional RT-PCR encompassing amplification and product analysis, which are two separate, sequential procedures (Wittwer et al., 1997). Generally, PCR product is loaded onto agarose or polyacrylamide gels for separation according to their size and then visualized with fluorescent dyes or

radioactivity. For real-time PCR, no post-PCR steps such as gel electrophoresis and ethidium bromide staining are required (Klein, 2002). This significantly reduces time and labor required for PCR product analysis. Therefore, it is suitable for large-scale analyses (Schena et al., 2004).

Another reason is that the LightCycler ™ PCR machine uses capillaries instead of tubes, which are heated by light instead of a heating block. This increasingly decreases the time needed to shift temperature in the process of real-time PCR. Also, the automatic recording during amplification in real-time PCR avoids collecting samples at different steps of a PCR experiment (Gachon et al., 2004).

1.6.2.1.3 Specificity

In contrast to techniques such as Northern blotting requiring hybridization of nucleic acids of several hundred base pairs long, real-time PCR guarantees a high specificity in the detection of a target sequence. This specificity is achieved by the use of two target sequence-specific short oligonucleotides (Gachon et al., 2004).

Since SYBR Green I non-selectively adheres to both the non-specific and specific products, this will affect the accurate quantification of target gene expression. However, this non-specific detection feature of SYBR Green I due to primer-dimers and other undesired products which have been frequently associated with real-time PCR assays can be overcome with the aid of a running ‘melting-curve’. A ‘melting curve’ is dependent on the GC content, length, and sequence and is obtained by monitoring the fluorescence of dsDNA dyes as the temperature passes through the product denaturation temperature (Wittwer et al., 1997). Gel electrophoresis and DNA sequence analysis can also be additionally used to give extra information about the specificity of the corresponding PCR product.

1.6.2.1.4 Accuracy

PCR generates copies of a DNA template in an exponential fashion. Real-time PCR amplification product is detected during the log-linear phase of a PCR reaction which is considered as the condition of constant amplification efficiency. Conventional PCR analysis requires gel electrophoresis of the end-point products. This may not yield consistent results since measurement of band intensity is very subjective (Munir and Kibenge, 2004). Furthermore, during the later rounds of amplification (the plateau phase) a PCR reaction is probably no longer generating products at an exponential rate due to various factors including reagent limitation, inhibitory PCR by-products such as accumulation of pyrophosphate molecules, and enzyme instability and competition between the DNA strands and primers for the complementary sequence (Ginzinger, 2002). Some reactions will generate more product than the others. Therefore, the conventional end-point for detection and identification of PCR products is unreliable in giving accurate quantification due to great variability in the efficiency of amplification than the real-time PCR reaction.

1.6.2.1.5 Practicability

SYBR is the simplest and least expensive chemistry that is commercially available. It is a brighter dye and can bind to double-stranded DNA in the minor-groove and exhibits increased fluorescence upon binding (Niesters, 2001). SYBR Green I was applied in real-time PCR (Schmittgen and Zakrajsek, 2000) for its higher affinity for any double-stranded DNA. It can be used for any type of sequence, regardless of the nucleotide nature. The incorporation of SYBR Green I into real-time PCR makes it a relatively economical advantage for testing and quantifying the expression of a large number of genes of interest.

1.6.2.2 Importance of internal controls used in real-time PCR

Quantitative real-time RT-PCR is one of the most sensitive techniques for detection of gene expression, particularly for low abundant mRNA. In order to get the accurate quantitative expression of the target gene, one or several internal control genes should also be included (Nicot et al., 2005) to account for any difference in RNA load due to differences in tissue mass, cell number, experimental treatment or RNA extraction efficiency (Schmittgen and Zakrajsek, 2000). Housekeeping genes are often used as internal standards as they are supposed to indicate the rate of transcription of genes which are not affected by experimental conditions.

Currently, at least 9 housekeeping genes are well described for the normalization of gene expression. The most common of these genes include actin, glyceraldehyde-3-phosphate dehydrogenase and ribosomal genes (Sturzenbaum and Kille, 2001). The expression of ideal internal control genes or housekeeping genes is presumed not to be affected by the experimental conditions. However, recent data suggest that expression of some housekeeping genes may vary with the extent of cell proliferation, differentiation and under various experimental conditions (Al-Bader and Al-Sarraf, 2005; Schmittgen and Zakrajsek, 2000). Therefore, selection of a suitable internal control gene is the first important step to successfully quantify the expression of genes of interest by real-time RT-PCR for different experimental conditions.

1.7 Regulation of flower senescence

1.7.1. Factors influencing flower senescence

Flower senescence is similar to leaf senescence, which may be divided into three phases: the initiation phase, the reorganization phase and the terminal phase (*Figure 1.3*). The onset of senescence is reported to be controlled by signalling cascades that initiate changes in gene expression and new protein synthesis (Hopkins et al., 2007).

The internal factors affecting plant senescence include age, developmental process and plant hormones (Chang et al., 2003; Guo and Gan, 2005; Rogers, 2006). With regard to

plant growth regulators in plants, ethylene plays a central role in developmental and physiological processes, such as seed germination, root initiation, growth, floral differentiation, flower senescence and the response of plants to environmental stresses (Arora et al., 2006; Chang et al., 1993; McGrath and Ecker, 1998; Sugawara et al., 2002; Wang and Kumar, 2004; Wang et al., 2003). According to Woltering, three types of flower life cessation were described (Woltering 1988). They are ethylene-sensitive petal wilting and withering, ethylene-insensitive petal wilting and withering, and ethylene-sensitive abscission of turgid petals. One of the characteristics of the first group is a sharp rise in ethylene production before visible petal wilting. Ethylene production was negligible at first, then increases and reaches a peak before beginning to decline again during senescence (Bartoli et al., 1996; Kuroda et al., 2003). It has been shown that following pollination, the ethylene produced in the stigmatic region serves as a mobile factor responsible for senescence symptoms observed in ovaries and petals (Llop et al., 2000; Wang et al., 2001; Woltering et al., 1995). In some non-climacteric flowers, ethylene is also produced and can mediate normal flower development and senescence processes (Setyadjit et al., 2004a). Abscission of florets in *Delphinium* is also caused by elevated levels of ethylene receptor, ERS1 (Kuroda et al., 2003).

However in some other species ethylene appears to play little or no part in flower senescence (Wagstaff et al., 2005; Wagstff et al., 2003). Ethylene-insensitive flowers do not appear to produce endogenous ethylene, or respond to exogenous ethylene treatments (Wagstaff et al., 2005). In iris flowers, the rate of respiration of the tepals remains unchanged, and that of ethylene production decreases during the process of tepal senescence. A negligible level of post-harvest ethylene production is observed in the flowers of *Sandersonia* (Eason and De, 1995). Ethylene-independent signals for senescence might be present in plants since even in ethylene-sensitive species the disruption of ethylene signaling or biosynthesis can delay, but not stop flower senescence.

Abscisic acid (ABA) is a possible candidate as a hormonal trigger for death of flowers that do not respond to ethylene (Panavas et al., 1998b). The increased level of

endogenous abscisic acid (ABA) has been observed in some flowers during petal senescence (Aneja et al., 1999; Panavas et al., 1998b). The longevity of rose flowers were negatively related to the level of endogenous ABA in the flowers at harvest (Garello et al., 1995). Application of exogenous ABA to detached rose flowers accelerated their senescence (Hunter et al., 2004; Muller et al., 1999). Leaf senescence enhanced by ABA may be due to interaction of ABA with other senescence inducers, for example ROS (Ferrante et al., 2004; Hung and Kao, 2004a). Petal abscission is generally sensitive to ethylene while ethylene-insensitive petal abscission also exists (van-Doorn, 2001). It is also not universally regulated by endogenous ethylene (van-Doorn, 2001; van-Doorn, 2002). It has been shown that application of jasmonate enhances plant senescence (Porat et al., 1993). The expression of a subset of senescence-related genes in *Arabidopsis* is enhanced by jasmonic acid application (Schenk et al., 2000). On the contrary, elevating levels in cytokinin, polyamine, and gibberellins have been reported to be the senescence inhibitors in some flowers (detail in the following section).

External factors such as temperature stress, drought, nutrient deficiency and pathogen infection have been reported to affect the time to senescence, perhaps by interacting with hormonal signals normally produced by the plants (Guo and Gan, 2005; Rubinstein, 2000). In flowers, mechanical stresses such as crushing the stigma or removing the stigma can accelerate petal senescence. This is associated with an increase in ethylene production (Ketsa et al., 2001). A limited amount of ethylene produced in sensitive species in response to the various environmental stress can ultimately lead to further increases in internal ethylene production prior to senescence (Jones and Woodson, 1999).

Nutrition deficiency is one of the reasons for senescence of the vegetative parts (Nooden et al., 1997). Removal of the reproductive 'sink' has been reported to delay the rate of vegetative tissue senescence (Srivalli and Khanna, 2004). Similarly, the removal of flowers in a cyme can lead to increased longevity of the first flower (Chanasuta U et al., 2003).

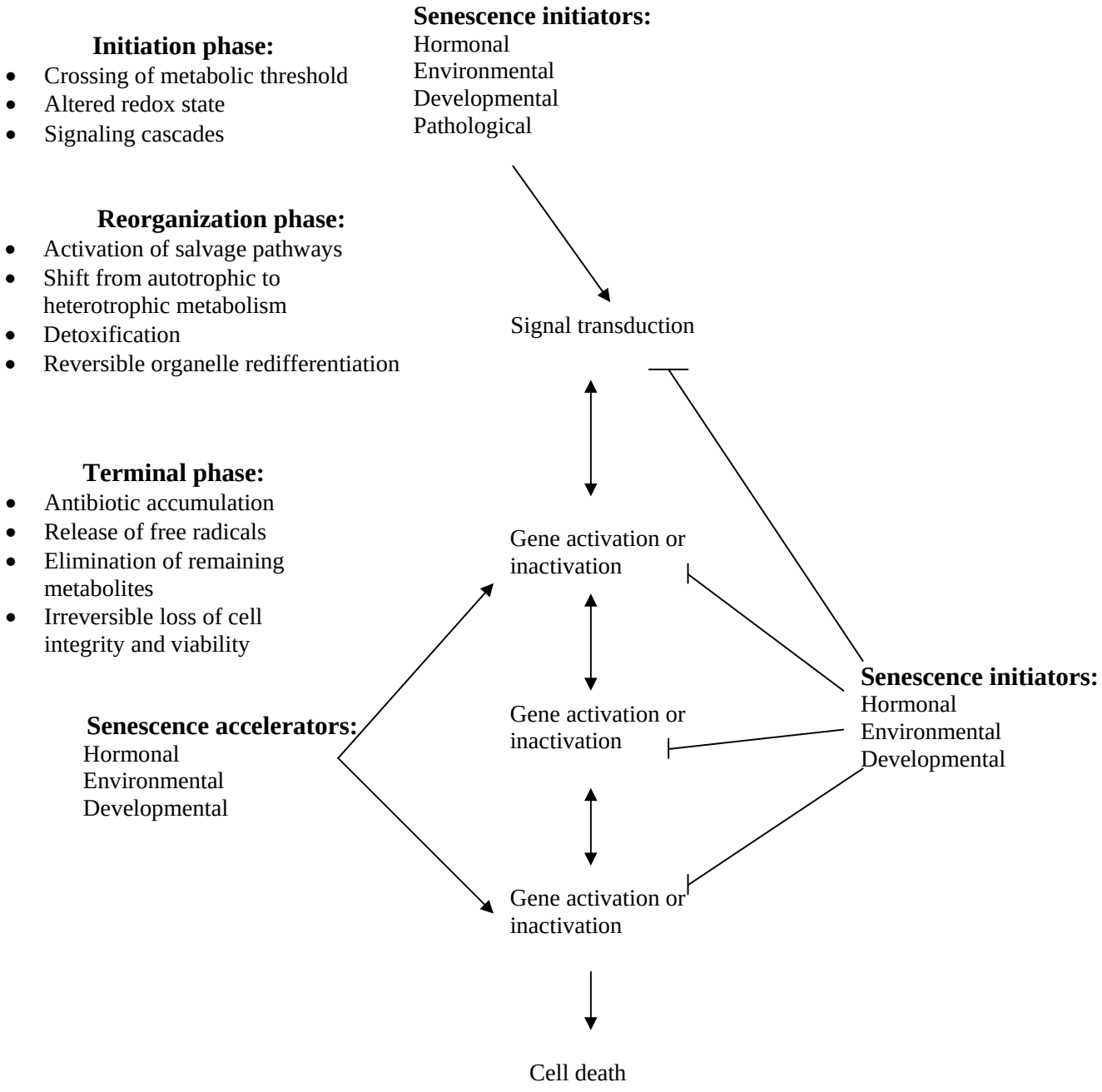


Figure 1. 3 A proposed scheme showing steps in the process of leaf senescence from the initiating signal to cell death (Buchanan et al., 2000).

1.7.2 Overview of post-harvest treatments to delay flower senescence

Although the post-harvest deterioration process of cut flowers cannot be reversed, some effective measures such as post-harvest treatments will probably maintain or slow this process. A bactericide added to vase water is often necessary to limit the growth of microbes that could otherwise block the water conducting tissues and affect water being taken up to keep the petals turgid. The following three factors are thought to have important anti-senescence effect on cut flowers.

1.7.2.1 Carbohydrate

Flower senescence is usually characterized by a decline in soluble carbohydrate content (Bieleski, 1993; Collier, 1997). Once detached from the plant and held under relatively low light intensities, flowers or leaves on the stems have little opportunity for photosynthesis. It seems likely that they do not have enough carbohydrates as respiration substrates and nutrients for the growth and development of the ovary and petal. A sugar added to a vase solution could play a very important role in keeping the quality of cut flowers (Ichimura and Hisamatsu, 1999; Ichimura et al., 2003). Sucrose pulsing or continuous sucrose treatment can reduce the rate of cut flower senescence and extend the vase life of cut flowers (Kim and Lee, 2002; Yakimova et al., 1996).

Except for its importance in energy generation, sugar added to a vase solution can play a role in various processes associated with plant growth and development including senescence. It can be used as materials for cell wall synthesis to promote floret opening, and it can increase anthocyanin concentration or prevent the up-regulation of senescence-associated gene expression (Burge et al., 1996; Hoeberichts et al., 2007; Ichimura, 1998; Zhang and Leung, 2001).

Sugars can play the role of an osmolyte in plant cells as well. The viability of the parenchymatous cells adjacent to vascular bundles was preserved better after trehalose

application in the petals of gladiolus spikes (Otsubo and Iwaya, 2000). Trehalose has also been reported to suppress the process of apoptotic cell death (Yamada et al., 2003).

Another mechanism of sugar regulation in plant senescence may be mediated through the regulation of hexokinase (HEX), the first enzyme in hexose metabolism. HEX functions as a sugar sensor to interrelate nutrients, light, and hormonal signaling networks for controlling growth and development (Moore et al., 2003). Transgenic tomato plants overexpressing HEX underwent rapid leaf senescence, suggesting that HEX may also be involved in senescence regulation (Dai et al., 1999). Overexpression of *Arabidopsis* or yeast HEX in plants results in early leaf senescence. Down-regulating *Arabidopsis thaliana* hexokinases delays the senescence process (Xiao et al., 2000).

1.7.2.2 Plant growth regulators

1.7.2.2.1 Cytokinin and senescence

Cytokinins are essential plant hormones with profound roles in growth and development, including senescence and longevity (Hwang and Sheen, 2001; Kim et al., 2006). Elevating cytokinin levels have been reported to delay flower senescence (Chang et al., 2003; Lukaszewska et al., 1994; Setyadjit et al., 2004b; Vaknin et al., 2005). However, in cut carnation, 2ip can delay the rate of flower senescence but do not prolong longevity (Van Staden et al., 1990). Dip treatment with BA could extend cut flower or inflorescence senescence (Huang and Chen, 2002). Senescence parameters including relative fresh weight, flower abscission, flower opening, flower discoloration and flower wilting were all suppressed by BA application (Setyadjit et al., 2004b).

During chrysanthemum flower senescence, the decrease in metabolic activity along with increased protein inactivation and degradation were delayed by BA application (Elanchezhian and Srivastava, 2001). In Mei flower (*Prunus mume* Sieb. et Zucc.), BA can inhibit flower abscission by regulating ethylene production (Guo and Sheng, 1999).

Another mechanism involving the anti-senescence effect of cytokinin probably is its influence on antioxidant defense system. The overproduction of cytokinins resulted in the stimulation of activities of antioxidant enzymes throughout the ontogeny of transgenic *Pssu-ipt* tobacco (Synkova et al., 2006). The isoenzyme profiles and intensity of antioxidant glucose-6 phosphate dehydrogenase were altered by kinetin treatment as well (Singh et al., 1997).

1.7.2.2.2 Polyamines and senescence

In plants, polyamines (PAs) have long been recognized to be linked with differentiation, growth, senescence, defense reactions and various environmental stresses (Guan et al., 2003; Li et al., 2006). It was reported that a freshly harvested fruit has a high level of polyamines, which decreased during fruit storage when membrane permeability and ethylene evolution increased (Jiang, 1995). The change on the ratio of endogenous spermidine (SPD) to putrescence (PUT) is associated with the onset of flower and fruit senescence (Lester, 2000; Serrano et al., 1991). Significant high linear correlations between total free polyamine levels and a senescence marker were found in the root nodule of *Vigna mungo* L. (Lahiri et al., 2004). Antisense expression of carnation cDNA encoding senescence-associated ACC synthase or ACC oxidase enhances polyamine level and abiotic stress tolerance in tobacco (Wi and Park, 2002). There is a positive correlation between polyamine content and induced senescence by oxidative stress from treatments with hydrogen peroxide (H_2O_2), high salinity, acid stress (pH 3.0), and ABA (Wi and Park, 2002).

Ethylene initiates ripening and senescence of climacteric fruits, whereas polyamines have been considered as senescence inhibitors (Tassoni et al., 2006a; Tassoni et al., 2006b). The senescence and programmed cell death of excised tobacco flowers were delayed by exogenous spermine treatment (Serafini et al., 2002). Application of polyamine improved quality of cut rose flowers and increased carnation flower longevity as well (Son and Chae, 1993; Yang et al., 2001b). Protein and RNA contents were significantly higher in polyamine-treated rose leaf discs. Peroxidase and cellulase activities were retarded by

polyamine treatments (Sood and Nagar, 2003). In spermine-treated cut roses, high levels of reduced sugars and soluble proteins were maintained, while the increase in membrane permeability, levels of MDA and ethylene evolution were inhibited (Yang et al., 2000).

Polyamines may bind to biological macromolecules, such as nucleic acid and proteins. They may stimulate DNA replication, transcription and translation. As antagonists of ethylene, they inhibit / delay physiological processes related to senescence (Niklas et al., 1998). There is a link between ethylene and polyamine metabolic pathways because of the common precursor S-adenosylmethionine (SAM) (Pandey et al., 2000; Tassoni et al., 2006a). Endogenous polyamines suppress ethylene production. They decrease endogenous 1-aminocyclo-propane-1-carboxylic acid (ACC) content, and the activities and transcript levels of ACC synthase and ACC oxidase in carnation petals (Lee et al., 1997). Unchanged ethylene production has also been found following spermidine treatment (Jiang, 1995). Polyamine is an effective scavenger of reactive oxygen free radicals, which can cause peroxidative damage to membranes and affect their integrity (Lester, 2000). Polyamines delay the process of senescence possibly by limiting lipid peroxidation and maintaining membrane function (Borrell et al., 1997; Jiang, 1995; Yang et al., 2001a).

1.7.2.2.3 Gibberellins and senescence

Gibberellic acid is generally regarded as a plant growth regulator that is associated with fruit ripening and senescence (Yu et al., 2006). In plants, it is reported that the level of GA declines prior to or during senescence (Guo and Sheng, 1999; Nooden and Leopold., 1988). GA can delay senescence in a variety of tissues, particularly in fruits and leaves (Chauhan and Bashist, 2001; Franco and Han, 1997; Jordi et al., 1994; Jordi et al., 1995; Kappers et al., 1997; Kappers et al., 1998; McDonald et al., 1997; Michalek et al., 2006; Whitman et al., 2001; Zaragoza et al., 1996).

In cut flowers, the application of GA_3 does not always delay flower senescence although a decline in endogenous gibberellins may be a correlative event associated with the onset of flower senescence as well (Saks and Van, 1993a; Saks and Van, 1993b). $GA_{(4+7)}$ can be used to prolong vase life, delay leaf senescence and enhance post-harvest quality of *Alstroemeria* cut flowers during their transport to market (Mutui et al., 2006). Likewise, Chrysal-SVB, a commercial pulsing solution (containing GA as one of its components), was effective to extend the vase life of *Cyrtanthus* flowers (Eason et al., 2002b). However, GA application does not delay senescence, but accelerated the rate of flower opening in *Prunus mume* Sieb. Et Zucc (Guo and Sheng 1999). Gibberellic acid can selectively extend the vase life or enhance the quality of some species of gentian, with a positive effect on two blue gentians but a negative effect on one white-colour species (Eason et al., 2004). Similarly GA application can suppress the post-harvest decline of membrane fluidity and the increase of electrolyte leakage in some rose cultivars (Sabehat and Zieslin, 1994). Although there are no effects on flower longevity of five rose cultivars tested, GA_3 can extend the longevity of detached petals in all of the cultivars tested (Goszczynska et al., 1990). The delaying of carnation flower senescence by gibberellic acid depends on the stage of flower development. Only if applied before petals reach full extension can GA treatment extend the time to complete petal vase life, indicating the requirement and importance of GA for bud opening and flower extension (Saks et al., 1992b).

The action of GA in plants is possibly related to its function on their antioxidant system. In *Pelargonium*, GA_3 acts to inhibit dark-induced leaf senescence by reducing ROS levels (Rosenvasser et al., 2006). During barley seedling establishment GA_3 can speed up senescence and death of aleurone cells (Bethke et al., 1999). The amounts of CAT and SOD (two enzymes that metabolize ROS) are greatly reduced in aleurone layers after GA application. However, treatment with GA in the presence of NO (nitric oxide) donors delays the loss of CAT and SOD (Beligni et al., 2002).

1.7.2.2.4 Inhibitors of ethylene biosynthesis or action

Attempts have been made to retard flower senescence and extend the vase life of pollinated flowers by applying inhibitors of ethylene biosynthesis or action (Arora, 2005; Ichimura and Goto, 2000; Singh and Moore, 1994). Aminooxyacetic acid (AOA) can extend the vase life of cut flowers by inhibiting ethylene production (Han and Lee, 1992; Kwack et al., 1996). Premature senescence caused by emasculation frequently encountered during the transport of cut flowers to the market was inhibited by 1-methylcyclopropene (1-MCP). This anti-senescence effect is related to complete inhibition of ethylene production and action (In et al., 2002; Son et al., 2002; Suh et al., 1999). STS is another known inhibitor of ethylene action which could block ethylene perception through the irreversible interaction of silver ions with ethylene binding sites. It can delay flower senescence or inhibit flower abscission by delaying or attenuating the increase in ethylene production (Guo and Sheng, 1999; Hansen et al., 1996). When cut carnation flowers were treated with silver thiosulphate, a high levels of water and carbohydrates in the petals was detected in the petals (Miura et al., 2000). Extending the vase life of cut rose flowers by STS was associated with a rise in total soluble sugars and a decrease in total free amino acids content in the petal tissues (Bhattacharjee and De, 1998). In gentian, an increased soluble carbohydrate level and decreased ethylene level are also associated with vase life improvement by STS (Zhang and Leung, 2001).

Application of ethanol may inhibit fruit ripening and increase flower longevity (Lee et al., 1999; Podd and van, 1999; Podd and van, 2002b; Pun et al., 1999; Wu et al., 1992). Ethanol could delay the senescence of open cut carnation flowers by inhibiting ethylene synthesis (Lee and Kim, 1999). The normal increase in ACC content, EFE activity as well as the flower sensitivity to exogenous ethylene were suppressed in ethanol-treated carnation (Wu et al., 1992). The application of low concentrations of ethanol may prevent normal climacteric respiration from occurring. High concentrations of ethanol may lead to increased membrane permeability and damages of the lipid bilayers, where the site of ethylene action is suspected to be (Podd and Van, 1998). However ethanol has been reported to promote oxidative stress, both by increasing ROS accumulation and

decreasing cellular defense in animals (Hoek et al., 2002). Ethanol also decreases general protein level. This could affect the metabolism of the flowers and prevent enzyme-mediated reactions as well as cell growth and development. The levels of chlorophyll and carbohydrate were decreased in carnation ovaries by ethanol application (Podd et al., 2002b).

1.7.2.3 Metabolic inhibitors

Protein degradation is a crucial mechanism in some developmental stages such as senescence and programmed cell death (Palma et al., 2002). An overall decline of protein level is always associated with a tissue undergoing senescence. One of the main reasons for this general trend is the large-scale proteolysis of cellular proteins (Collier, 1997). However, reduced synthesis of proteins may also contribute to this reduction of protein level.

Various inhibitors can be used for preventing the degradative processes in cells during senescence. Since there is evidence for the synthesis of certain new proteins in tissues undergoing senescence (Eason and De, 1995; Eason et al., 2002d), some protein synthesis inhibitors have been found to delay senescence of plant organs. In iris flowers large-scale protein degradation is associated with visible senescence. Two unspecific inhibitors of serine protease considerably delayed iris flower senescence (Pak and van, 2005). It is found that cysteine protease inhibitors, 2,2'-dipyridyl, also delayed flower senescence of *Sandersonia aurantiaca* Hook (Eason et al., 2002a). However, the inhibition of protein degradation is not always correlated with a delay in the time to senescence. Phenylmethylsulfonyl fluoride (PMSF), a protease inhibitor, had a similar inhibitory effect on protein levels but did not affect the time to visible senescence. Also several other protease inhibitors did not affect the time to iris tepal senescence (Celikel and Van, 1995).

Cycloheximide (CHI), a non-specific inhibitor of the peptide transferase activity of the 60s ribosomal subunit, is frequently used to prolong the vase life of cut flowers. This is

probably related to a retardation protein content decline and maintaining the protein population as visualized by SDS-PAGE (Lay et al., 1992). Treatment of daylily buds with cycloheximide as the flowers commenced opening delayed the onset of visual signs of senescence (Courtney et al., 1994). In addition, petal opening and senescence of cut *Gladiolus, Iris,* and *Narcissus* are significantly inhibited by continuous treatment with CHI (Dumont et al., 2002).

In *Leptospermum scoparium* Forst, CHI treatment resulted in a marked reduction in the rate of decline of water uptake and loss of leaf moisture content (Burge et al., 1996). Cycloheximide treatment also inhibited the loss of pigment or colour fading associated with ethylene-insensitive sandersonia flower senescence, implicating that protein changes are required for pigment degradation in this system (Eason and De, 1995).

Since increased RNase synthesis and ROS accumulation are associated with the process of senescence, transcription inhibitors and free radical scavengers can affect petal senescence as well. For example, actinomycin D, an inhibitor of transcription, has been reported to suppress the onset of visible petal senescence (Yamada et al., 2007b). Treatment with a-amanitin, an inhibitor of DNA-dependent RNA synthesis, also substantially delays the onset of flower senescence (Jiang et al., 2007). Nitric oxide (NO.) is a signalling molecule, a pro-oxidant, and a potential effective chain-breaking antioxidant in free radical-mediated lipid oxidation (Hummel et al., 2006). It has been shown that NO can remove ROS including hydrogen peroxide in peroxisome (del Rio et al., 2003). 2,2'-(hydroxynitrosohydrazino)-bisethanamine, a NO donor compound, has a widespread applicability to cut flowers since it has been shown to prolong the vase life of eight flowers (Badiyan et al., 2004). However, in some flowers, free radical scavengers / antioxidants do not affect the time to visible tepal senescence (Bailly et al., 2001).

1.8 Thesis approach

1.8.1 Aims and objectives

Senescence is a developmentally regulated programmed process, which is hypothesized to be governed at the genetic level. However, some external and internal factors might affect the natural senescence process due to their influence upon the central mechanism of senescence. Currently, silver thiosulfate (STS) is widely used as a post-harvest treatment to maintain the keeping quality of freshly cut flower stems. However, STS is now recognized as a potential environmental hazard. It is a matter of time before most, if not all, countries that import cut flowers will ban the use of STS. The anti-senescence effect of STS seems to result from its interference with some key regulatory events in flower senescence, including ethylene production.

The proposed study aimed to explore some potential post-harvest treatments for cut flowers. Several chemicals including plant growth regulators, carbohydrates and ROS scavengers were investigated. Furthermore, potential anti-senescence mechanisms were investigated in order to increase our understanding of the critical events leading to senescence.

In preliminary experiments, four post-harvest treatments (sucrose, GA, ethanol and STS) were found to be effective in delaying flower senescence of gentian and petunia. Their anti-senescence effects are commonly reported to be correlated with the pathway of ethylene. This study sought to obtain new insights into other poorly characterized aspects of these post-harvest treatments on senescence of gentian and petunia flowers. Particularly, the changes in certain oxidative / antioxidant events in response to selected chemical treatments in cut gentian and petunia flowers were studied.

The main objectives of this project were:

- Screening for possible anti-senescence chemicals by determining the post-harvest life or petal wilting of cut gentian and petunia flowers in response to treatments with these chemicals.

- Investigating the biochemical, ultrastructural and molecular changes in response to selected chemical treatments.

- Revealing the possible involvement of the antioxidant defense system in cut flowers of gentian and petunia in response to the appropriate chemical treatments.

1.8.2 Thesis structure

This thesis consists of five chapters. Chapter 1 is a general introduction, which describes the research background, objectives and thesis structure. The materials and methods used in this study are described in Chapter 2. The findings on the effects of anti-senescence chemicals and their possible mechanisms in cut gentian and petunia flowers are presented in Chapter 3 and 4 respectively. In Chapter 5, an overview of the major findings, further discussion, conclusions and recommendations for further studies is provided.

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant materials

2.1.1 Gentian (*Gentiana triflora,* Pall. variety axillariflora)

Freshly cut gentian stems were harvested, placed directly into buckets of water and transported to the laboratory from a commercial gentian farm, which is about 5 km from the University of Canterbury, Christchurch, New Zealand. *In vitro* flowers from the *in vitro* gentian plants cultured as described previously (Zhang and Leung, 2001) in the plant biotechnology laboratory at the University of Canterbury, were used in some experiments, particularly during the preliminary trials. Briefly, nodal cuttings of *in vitro* gentian plantlets were cultured on double-strength WPM medium, containing 3% (w/v) sucrose, 0.75% (w/v) agar and 0.2 mg/L BA for 12 weeks. The pH of the medium was adjusted to 5.5 with 0.1 M NaOH before medium sterilisation in an autoclave.

2.1.2 Petunia (*Petunia hybrida,* variety 'Hurrah')

Detached petunia flowers used in this study were collected from plants grown in the glasshouse at the University of Canterbury, Christchurch, New Zealand. For continuous propagation of the petunia plants, several small branches (with three or four leaves) were grown in a potting mix containing 60% bark, 20% peat and 20% sterilized soil for a period until the root system was growing well enough. Then, they were transferred individually into pots containing the same potting mix. The cuttings were maintained under natural light in the glasshouse at 25-30˚C / 15-20°C day/night during summer

months and 25-30˚C / 15-20°C day/night and under 16-hour photoperiod (supplemented with sodium light, 400W) during winter months. Flowering normally began 1-2 months after a rooted cutting was transferred to a pot but took longer in winter. Plants more than 5 months old were discarded and not used in this study.

2.2 Post-harvest treatments: chemical manipulation

2.2.1 Post-harvest treatments of flowers borne on cut gentian stems

Cut gentian stems were trimmed just before start of a post-harvest treatment to a length of 60 cm. Then leaves on the lower part of the stems (about 20 cm from the cut ends) were removed before the cut stems were put into 500 ml reagent bottles containing 200 ml of various test solutions or distilled water.

In the first set of experiments, gentian stems were harvested when about 40% of the buds on the stems were open (at Stage 5, see *Plate 2.1*). They were treated for 24 hours at about 22 ºC with one of the following five solutions: distilled water, sucrose (3%, w/v), gibberellic acid (GA_3, 5.0 µM), spermine (Spm, 0.5 mM), kinetin (Kin, 0.3 µM) and cycloheximide (Cyclo, 0.1 mg/L). There were 10 replicate stems in a treatment.

In further experiments, less mature stems (fewer than 3 flowers on the stems had reached Stage 5) were collected and pulsed at about 22 ºC for 24 hours with either distilled water or sucrose (Suc) (3%, w/v). The anti-senescence effect of GA_3 in gentian petals was also evaluated following pulsing for 24 hours with different concentrations of GA_3. In addition, the effect of a combination of GA_3 (5 µM) with sucrose (3%) was assessed. Each treatment was replicated with nine stems.

After pulsing, the stems were transferred to one litre Erlenmeyer flasks containing 500 ml distilled water with 200 mg/L 8-hydroxyquinoline sulphate (8-HQS) as a germicide. The flowers borne on the treated gentian stems were inspected daily and the vase life of each flower was assessed as described in Section 2.3.

2.2.2 Treatment of detached flowers and isolated half petals of gentian with GA_3

Detached blue buds (Stage 3) or open flowers (Stage 5 and/or Stage 6 flowers) were subjected to post-harvest treatments. Each treatment consisted of pulsing a blue bud or open flower in 3 ml each of different concentrations (1, 5 or 10 µM) of GA_3 or distilled water for 24 hours. Isolated half gentian petals were pulsed in distilled water or 5 µM GA_3 for 24 hours. There were 5 replicate blue buds or open flowers or half petals in a treatment. After pulsing, the floral parts from a treatment were placed in a glass vial and their basal parts were immersed in 25 ml distilled water containing 200 mg/L 8-hydroxyquinoline (8-HQS). However, only three individual flowers were used as replicates per treatment to assess changes in enzyme activity.

Plate 2. 1 Stages in the development and senescence of gentian flowers

The following chronological stages of development and senescence of gentian flowers were distinguished from left to right: Stage 1, Green bud (GB); Stage 2, Blue green bud (BG); Stage 3, Blue bud (BB); Stage 4, Opening bud (OB); Stage 5, Just opening flower (JOF); Stage 6, Opening flower (FLO): tip of the flowers became wide open; Stage 7, Starting senescence (SS): the blueness of the petal beginning to fade away; Stage 8, Half-

senescence (HS): blue petals starting to become brown; Stage 9, Fully senesced flower (FS): most of the petal had turned brown.

2.2.3 Post-harvest treatments of petunia flowers

Petunia flowers or petals (Stage 7 or 8, see *Plate 2.2*) were harvested and used immediately for experiments. Individually the stalk of isolated petunia flowers or the basal part of petal was inserted and held continuously in 2, 4 and 6% (w/v) ethanol or distilled water in glass vials covered with aluminium foil paper.

Plate 2. 2 Stages in development and senescence of petunia flowers

From left to right: 1, Small bud (B); 2, Just elongating bud (JEB); 3, Elongated bud (EB); 4, Opening bud (OB); 5, Just opening flower (JOF); 6, Half-open flower (HF); 7, Fully open flower (OF); 8, 'Beyond open flower' (BO): fully open flower that had become larger; 9, Pre-senescence flower (PS): flowers lost signs of freshness; 10, Starting

senescence flower (SS): flowers starting to show visible signs of senescence-about 25% of the petal edges wilted; 11, Half-senesced flower (HS); 12, Fully senesced flower (FS).

2.3 Flower vase life assessment

2.3.1 Gentian

After different pulsing treatments, the plant materials were placed in a growth room kept at 22 ºC, 70% relative humidity, and 16 hours of illumination daily at a photosynthetic photon flux (PPFD) of 60 umol · m^{-2} · s^{-1} using cool white fluorescent tubes. Vase life evaluation was started from the time flowers were put into the appropriate test solutions. The following parameters were monitored: the bud color changes, bud opening and senescence of flowers that had opened.

Vase life of flowers on the stems which were put in a test solution at the bud stage was assessed only when they had become open. The percentage of flower senescence on a stem was calculated by dividing the number of flowers that showed visible signs of senescence with the total number of flowers on the stem. The end of vase life of the flowers on each stem was defined as the time when more than 50% of the florets reaching maturity had wilted.

2.3.2 Petunia

Vase life of an individual flower after a post-harvest treatment and kept in a growth room as described in Section 2.3.1 was considered terminated once the petal noticeably wilted or when the petals began to lose turgor.

2.3.3 Flower fresh weight measurement

Fresh weights of detached flowers or petals of gentians and petunia were determined at specific days following post-harvest treatments as part of vase life assessment.

2.4 Collecting petals after start of post-harvest treatments

At a specific day after initiation of ethanol or GA treatments, at least three petals from each treatment as a replicate sample were pooled together, immediately frozen in liquid nitrogen and stored at -80ºC for later physiological, biochemical and molecular analyses.

2.4.1 Electrolyte leakage measurement

Harvested gentian or petunia petals were cut into small pieces and then placed in 30 ml distilled water. The conductivity of the solution was measured using a conductivity meter (HI 8733, Singapore) after a 3-hour incubation period on an SS70 orbital shaker (Chiltern Scientific, New Zealand) at about 22 ºC. After the solutions were autoclaved for 2 hours and cooled to room temperature, the conductivity was measured again. Results are expressed as % of total leakage from petals autoclaved for 2 hours in water and represent the means of 3 replicates ± SE.

2.4.2 Cell sap pH determination

Petunia petals from different developmental stages were ground in distilled water at room temperature. After the homogenates were centrifuged at 4˚C for 20 min at 11,000 rpm, the supernatants obtained were used for determination of cell sap pH (correlating to changes in vacuolar pH) using a pH meter (E350B, Swizerland).

2.5 Biochemical analysis

2.5.1 Preparation of crude enzyme extracts

Firstly, gentian or petunia petals were frozen in liquid nitrogen and were then homogenized to a fine powder in a cold mortar. Then, soluble proteins and enzymes were extracted using 50 mM potassium phosphate buffer (pH 7.0) containing 1% (w/v) insoluble polyvinylpolypyrrolidone (PVP) in a ratio of 1.0 g tissue to 5 ml buffer. Extracts for determining ascorbate peroxidase were prepared as described above, except that 0.5 mM ascorbate was also added to the enzyme extraction buffer to prevent inactivation of ascorbic acid peroxidase. The homogenates were centrifuged at 4˚C for 20 min at 11,000 rpm. The volumes of the supernatants obtained were measured before they were stored at -80˚C for later use in determination of protein and enzyme activities.

2.5.2 Determination of total soluble protein content

Total protein concentration of petunia and gentian petal extracts was determined according to Bradford (1976). Petal extracts (0.1 ml each) were mixed with 1 ml of Bradford reagent, and incubated for 5 min at room temperature before the absorbance was measured at 595 nm (SmartSpec Plus spectrophotometer, BioRad, California). A standard curve was prepared with bovine serum albumin (BDH, England).

2.5.3 Determination of antioxidant enzyme activities

Total superoxide dismutase (SOD EC 1.15.1.1) activity was measured spectrophotometrically according to Dhindsa et al. (1982), which was based on the ability of an enzyme extract to inhibit the photochemical reduction of nitroblue tetrazolium. The reaction mixture (1 ml) contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 0.1 mM EDTA, 75 µM NBT and 2 µM riboflavin. Control was prepared in an identical manner using a portion of petal extract that was first heated for 10 min in a boiling water bath. After illumination in a plant growth room under a cool white fluorescent tube for 10 min, absorbance was measured at 560 nm. One unit of SOD was defined as the amount of a petal extract that brought about half-maximum inhibition of the photoreduction of NBT to blue formazan. SOD activity of the extracts was expressed as units of SOD per min per mg of total petal protein, per fresh weight or petal.

Ascorbate peroxidase (AP EC 1.11.1.11) activity was determined essentially according to Rao et al. (1996) by following a decrease in ascorbate concentration measured at 290 nm for 10 mins. The reaction mixture consisted of 0.5 mM ascorbic acid, 0.2 mM H_2O_2, 0.1 mM EDTA and 50 mM sodium phosphate buffer (pH 7.0). Correction was made for the non-enzymatic oxidation of ascorbate by H_2O_2. AP activity was expressed as the decrease of absorbance at 290 nm per min per mg of total protein or fresh weight or petal.

Non-specific peroxidase (POD EC 1.11.1.7) activity was measured at 470 nm for 10 min in a reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0), 5 mM pyragaoll, 2 mM H_2O_2 and 10 µl enzyme extract. The enzyme activity was expressed as the increase in absorbance at 470 nm per min per mg of total protein, per fresh weight or per petal. The values obtained in the absence of enzymatic extracts or substrates were used for corrections. The enzyme assay was run in triplicates and the mean value was obtained as the result.

2.5.4 Ascorbate determination

One gram of petunia petals were homogenized in 5-10 ml of meta-phosphoric acid, centrifuged for 20 min at 10000 rpm at 4˚C. The ascorbate (ASC) concentration in the supernatant was quantified spectrophotometrically according to the method described by Bartoli et al. (1997a) following the reduction of the oxidized ascorbate forms with dithiothreitol (DTT). The reaction mixture (1.2 ml) contained 0.3 ml of supernatant, 100 mM Na_3PO4 (pH 7.4), 3 mM EDTA and 1.25 nM DTT. After 10 min of incubation at 20 ˚C, 0.15 ml of 0.5% N-ethylmaleimide (w/v) was added to remove excess DTT. For control**,** water was substituted for DTT and N-ethylmaleimide. Color was developed by adding 0.6 ml of 10% (w/v) TCA, 0.6 ml of orso-phosphoric acid, 0.6 ml of 4% a, a-dipyridyl in 70% (v/v) ethanol and 0.3% (w/v) $FeCl_3$. The changes of A_{525} were read after a 40 min of incubation at 40 °C (Bartoli et al., 1997a). A standard curve was generated using absorbance values at 525 from a series of concentrations of ascorbate. Then ascorbate levels in petal tissues were calculated according to the linear regression

line obtained by plotting the ascorbate concentration against the corresponding absorbance value.

2.5.5 Determination of chlorophyll and carotenoid levels

Nine gentian and petunia petals were rapidly ground to a fine powder in a mortar using liquid nitrogen. Chlorophyll and carotenoid were extracted with 85% (v/v) acetone. The homogenates were centrifuged at 10,000 rpm for 15 mins at 4°C and the supernatants were used for determination of chlorophyll and carotenoid levels. These extracts were kept on ice in the dark to minimize pigment degradation and assayed spectrophotometrically as soon as possible. The contents of chlorophyll a, b and carotenoids in crude acetone extracts of petals were calculated according to the following formulae, which were based on the report in the handbook of photosynthesis (Pessarakli, 1997).

Chlorophyll a (μg) = $(12.21 \times OD_{663\ nm} - 2.81 \times OD_{646\ nm}) \times$ Volume (ml)

Chlorophyll b (μg) = $(20.13 \times OD_{646\ nm} - 5.03 \times OD_{663\ nm}) \times$ Volume (ml)

Total chlorophyll (μg) = chlorophyll a + chlorophyll b

Carotenoids (μg) = $4 \times OD_{480\ nm} \times$ Volume (ml),

The chlorophyll or carotenoid contents of petal tissues were expressed as per petal or fresh weight basis.

2.5.6 Anthocyanin extraction and quantification

Gentian petals were grounded to a fine powder with a pestle and a mortar using liquid nitrogen. Anthocyanins were extracted with 1% (v/v) HCl in methanol according to the method of Weiss and Halevy (1989). The anthocyanin extract was centrifuged at 11,000 rpm for 10 min at 4 °C, and the supernatant was used as a source of the pigment. The

concentration of anthocyanin in gentian petals was expressed as absorbance at 530 nm per gram fresh weight of gentian petal tissue (Weiss and Halevy, 1989).

2.5.7 Detection of superoxide anion and hydrogen peroxide in petal tissue

Detection of H_2O_2 and $O_2^{\cdot-}$ in petunia petals was performed using the methods of the following authors with some modifications (Fryer et al., 2002; Wohlgemuth et al., 2002). Superoxide accumulation in petal tissue was determined with nitro blue tetrazolium (NBT), which reacts with superoxide, to produce a blue formazan precipitate. Petal tissue was also incubated in a solution containing both NBT and 10 mM $MnCl_2$, a highly effective superoxide dismutaing catalyst agent, as control. For detection of H_2O_2, o-dianisidine (DAS) was used as substrate which was polymerized to form a deep brown product in the presence of H_2O_2 in petal tissues. As control petal tissue was incubated in the presence of the substrate and 10 mM ascorbic acid, a hydrogen peroxide scavenger.

Whole petals were carefully separated from the green sepal before being to water at the petal base. Then the petals were pulsed in STS solution for 24 hours. Also some petals were held in ethanol or water. On a specific day, in a petridish (9-cm diameter) petals from these treatments were incubated in 0.1% (w/v) NBT, 10 mM sodium azide, 50 mM potassium-phosphate, pH 6.4 for 8-10 hours in the dark or in 0.1% (w/v) DAS, 10 mM 2-(N-morpholino) ethanesulphonic acid (MES) for 18-36 hours in the light. Part of the petal tube was cut off so that the lid of the petridish could be placed on the top of the dish properly.

2.5.8 Protein gel electrophoresis and isozyme analyses

2.5.8.1 SDS PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) for proteins

Electrophoresis on denaturing polyacrylamide gels was performed on a BioRad mini vertical slab gel system (Mini Protean II) based on the method of Laemmli (1970). Ammonium persulphate (10%, w/v) and TEMED were used as catalyst and initiator respectively for the polymerization of 12% separating gels and 4% stacking gels. The gels were prepared according to the instructions of the manufacturer of the Mini Protean II System (BioRad, California, USA).

Proteins from petunia petals were extracted using a sodium phosphate buffer (50 mM) at pH 7.0 as described in Section 2.5.1, except that the ratio of tissue/buffer changed from 1/5 to 1 tissue / 3 ml of buffer. Four volumes of a protein extract were first mixed with one volume of SDS-PAGE sample buffer, then boiled for 5 min and centrifuged. After this, a known volume of this protein extract was loaded into a well. Low range SDS-PAGE Molecular Weight Standards were run concurrently with petal samples on the gels. Gels were run using Tris-glycine buffer (pH 8.4) at 200 V for 40 min at room temperature.

2.5.8.2 Non-denaturing PAGE for analysis of antioxidant enzyme activities

Enzyme extracts from flowers of different stages or the ethanol-treated petunia petals were subjected to discontinuous PAGE, using a Mini Protean II vertical slab gel system (BIO-Rad, California, USA). The gels were of similar composition to those for SDS-PAGE except with the omission of SDS. They were run as described following the instructions of the manufacturer of the Mini Protean II Vertical Slab Gel System.

A sample for loading was prepared by mixing two volumes of an enzyme extract with one volume of 60% (w/v) sucrose. Then, 10-15 µl of this was loaded onto a well in a gel. The boiled samples were used as the controls in preliminary experiments. The gel running condition was also similar to SDS-PAGE except that the carrier buffer contained

2 mM ascorbate. Gels were pre-run for 30 min to allow ascorbate to enter gels prior to application of petal extracts containing the AP isozymes.

2.5.9 Protein staining procedures

2.5.9.1 Commassie blue staining

After SDS-PAGE, gels were stained for 4 hours at room temperature, while being shaken (20 r.p.m.); in about 100 ml of Commassie blue stain solution consisting of 50% (v/v) absolute methanol, 10% (v/v) glacial acetic acid and 0.1% (w/v) Commassie Blue R 250. They were destained with 5% (v/v) methanol and 10% (v/v) glacial acetic acid at room temperature overnight or until the bands became visible following several changes of destaining solution.

2.5.9.2 Silver staining

Silver staining of SDS-PAGE gels was carried out basically according to Suo (2000). Following SDS-PAGE, gels were fixed in 50% (v/v) methanol and 10% (v/v) glacial acetic acid solution for 1 hour. After four 5-min rinses with distilled water, they were incubated in 10% (v/v) glutaraldehyde for 30 min. Then 4 rinses 15 min each in distilled water were performed to remove glutaraldehyde. Following another overnight washing, the freshly made silver staining solution containing 0.7% (w/v) AgNO3, 1% (v/v) ammonia solution and 0.08% (w/v) NaOH was added. After staining for 15 min, gels were washed with 4 changes of distilled water 5 min each time. Finally colour development was initiated in a solution consisting of 0.01% (w/v) citric acid and 0.1% (v/v) formaldehyde. When the bands were clearly visible, the colour developing process was stopped by putting the gel in 5% (v/v) acetic acid. All the steps were carried out with shaking at 20 rpm at room temperature.

2.5.10 Antioxidant enzyme activity staining

After electrophoresis, SOD isoforms were visualized by incubation of non-denaturing gels in 50 mM sodium phosphate buffer (pH 7.0) containing 0.24 mM NBT and 28 µM riboflavin for 20 min in the dark and then immersing the gels in 28 mM TEMED, followed by exposure to a light source at 25°C in a growth room for 10-20 min (Dalal et al., 2001).

Following the method of Dalal et al. (2001), staining for POD isoforms was achieved by incubating gels for 15-20 min in 200 mM sodium acetate buffer (pH 5.0) containing 2 mM benzedine and 3 mM H_2O_2.

After non-denaturing PAGE, APX was detected following the method described by Dalal et al. (2001). Gels were placed in 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 40 min. Then they were incubated in the same buffer containing 4 mM ascorbate and 2 mM H_2O_2 for another 20 mins. APX isoforms were visualized by submerging the gels for 15-30 min in sodium phosphate buffer (pH 7.8) containing 28 mM TEMED and 2.45 mM NBT. Controls included non-enzymatic (buffer only) and heat-denatured enzyme (boiled for 10 min) samples.

2.6 Ultrastructural analysis of gentian petals

2.6.1 Sample fixation and embedding

Approximately 2×5 mm^2 segments were excised transversely from petals, immediately fixed in glass vials containing 3% (v/v) glutaraldehyde and placed under vacuum for overnight infiltration. Following three 10-min rinses with phosphate buffer, the petal tissues were post-fixed in 1% (v/v) osmium for 3 hours and subjected to a 10-min infiltration rinse with phosphate buffer. Then the petal tissues were passed through a

dehydration series of acetone (20-80%), 10 min each step and followed by three times of 100% acetone soaking.

A Spurr resin / 100% acetone (1:2, v/v) mix was used for embedding the petal tissue. After overnight incubation in a continuous, circular rotating agitator, the tissues were left for three hours rotating in a Spurr resin /100% acetone (2:1, v/v) mix. Finally, the tissues were transferred to small plastic caps, immersed in 100% Spurrs resin and baked for eight hours at 65 °C in a bench top laboratory oven. Then the solid resin disks were removed from their caps and stored at room temperature for later uses.

2.6.2 Preparation for transmission electron microscopy

The prepared solid resin disks of petal tissues were cut and affixed to a resin stud. Then the petal tissue block was clamped onto an ultramicrotome via the attached resin stud. Surplus resin was trimmed from each resin block with a razor blade to create a flat sectioning surface. The flat surface was aligned with an ultramicrotome glass blade, with which the block was trimmed until the specimen could be completely visualised in each section. After staining with uranyl acetate and lead citrate, the specimens were mounted on grids and viewed under a transmission electron microscope (TEM).

2.7 Cu-Zn SOD gene expression analysis

2.7.1 RNA preparation

2.7.1.1 Isolation of total RNA

According to the manufacturer's procedure, total RNA was extracted from petunia petals using Trizol reagent (Invitrogen, Life Technologies, New Zealand). About 4-5 petunia petals were ground in liquid nitrogen using a cold mortar and pestle. Then 200 mg

homogenized tissue was transferred to a sterilized Eppendorf tube which was stored temporarily in liquid nitrogen until use.

After all samples were weighed, in order to lyse tissue one ml Trizol reagent was added to each sample tube, which was then vortexed and incubated at room temperature for 10 min. Then chloroform (200 µl) was added to the tube, vortexed vigorously and incubated for 10 min at room temperature. The phases were separated by centrifugation at 14,000 rpm for 15 min at 4 °C. The upper aqueous phase was transferred to a new tube and care was needed to transfer as little of the thick interface as possible. Then 500 µl ice-cold isopropanol was added and the mixture was vortexed, stored at room temperature for 10 min and centrifuged at 14000 rpm for 10 min at 4 °C. After centrifugation, the supernatant was discarded.

The RNA pellet was washed with one ml ice-cold 75% ethanol and centrifuged at 7500 rpm for 7 min at 4 °C. After ethanol was removed, the pellet was air-dried for 10-15 min, dissolved in 30-50 µl sterile DEPC-treated water and stored at -80 °C until use.

2.7.1.2 Quality evaluation of isolated RNA

The isolated RNA samples were diluted 40-100 times and the quantity and quality (purity) of the RNA were assessed spectrophotometrically. The ratio of $OD_{260\ nm}$ / OD_{280} nm was used to evaluate the purity of the RNA sample. The value of the ratio for pure RNA is 2:1. A ratio of 1.7-2.0 indicates that RNA preparation is of high quality (Dr. Jason Song, personal communication). Agarose gel (1%, w/v) electrophoresis was also performed to assess the integrity of each RNA sample.

2.7.1.3 Quantification of isolated RNA

The concentration and yield of each RNA sample were determined by calculation according to the following formula:

RNA content (µg/µl) = 40 (µg/ml) × $A_{260\ nm}$ × dilution factor / 1000

Total RNA yield (µg/petal) = 40(µg/ml) × $A_{260\ nm}$ × dilution factor × volume of tissue (µl) / number of petal

2.7.2 Reverse transcription for cDNA synthesis

Total RNA isolated from petunia petals on day 0, 3 and 6 after ethanol and water (control) treatments was used for cDNA synthesis. Firstly 1 µg total RNA was subjected to DNAase I (New England Biolabs, Massachusetts, USA) treatment at 37 °C for 10 min following the manufacturer's instructions in a Programmable Thermal Controller (PTC-100, MJ Research, UK). DNase was denatured at 75°C for 10 min after1 µl EDTA was added. RNA was precipitated with isopropanol, washed with 75% ethanol and finally dissolved in 10 µl DEPC-water with 1-fold RNAsecure (Ambion, Austin, USA). To activate the ability of RNAsecure to protect RNA mixture was heated at 65 °C for 10 min. According to the manufacturer's instructions, first-strand complementary DNA synthesis was conducted by incubating 1 µg RNA, 1µl Oligo-$p(dT)_{15}$ primer and 1 µl Random primer $p(dN)_{6.}$ After the secondary structure of RNA was denatured at 65°C for 10 min, the mixture was put immediately on ice for 5 min. Then, 6.5 µl master mix (4 µl 5-fold RT buffer, 0.5 µl RNase inhibitor 40 units / µl, 2 µl 10 mM dNTPs) and 0.5 µl transcriptor reverse transcriptase were finally added to each cDNA synthsis mixture (a total volume of 20 µl).

This was incubated firstly at 25 °C for 10 min, and then at 55 °C for 30 min. The reaction was stopped by heating at 85 °C for 5 min. The synthesized cDNA was stored at -20 °C

for subsequent PCR analysis. Negative control (without reverse transcriptase) was used to determine the presence of contaminating genomic DNA.

2.7.3 Real-time RT-PCR

2.7.3.1 Real-time PCR amplification condition and program setting

PCR primers used in this study were designed based on the homologous gene sequences of related species downloaded from the GenBank database (Appendix B 1) using the Primer Premier 5 Software. They were synthesized chemically by Invitrogen Life Technologies (New Zealand), and their sequences are listed in Appendix B (1).

The mRNA levels of target and reference genes were quantified using real-time PCR with the FastStart SYBR Green Master kit (Roche Diagnostics, Germany) following the manufacturer's instructions. Each 20 µl PCR reaction mixture contained 1 µl of cDNA template, 1 µl of each forward and reverse primer (18 µM), 10 µl of FastStart SYBR Green Master, and 7 µl sterilized distilled water. Real-time PCR reaction was conducted in a thermal cycler (Mx3000P TM real-time system, Stratagene, USA) with the following thermal cycling conditions: initial 95 °C for 10 min to activate the Taq DNA polymerase, followed by 45 cycles of denaturation for 30 s at 95 °C, annealing for 20 s at 55 °C and extension for 30 s at 72 °C. Fluorescence data were acquired during an extra 10 s at 74°C following the extension phase.

2.7.3.2 Standard curve of Cu-Zn SOD gene amplification

Standard curves were generated using the fluorescence data using a series of diluted cDNA (1-, 10-, 100-, 1000- and 10000-fold) as PCR templates. The Ct values were subsequently used to calculate and plot a linear regression line by plotting the logarithm of template concentration against the corresponding threshold cycle.

2.7.3.3 Determination of PCR amplification efficiency for Cu-Zn SOD and reference genes

The PCR amplification efficiency of a gene was calculated based on the slope of the standard curve according to the equation:

$$E = 10^{(-1/slope)} - 1$$

Where E is the amplification efficiency of the PCR reaction

S is the slope of the standard curve.

The accuracy of the PCR amplification efficiency can be judged by the performance of the standard curve. 100% PCR amplification efficiency means that the amount of template is doubled after each cycle, as demonstrated by the slope of the standard curve equation (Mocellin et al., 2003). The slope value and correlation coefficient of the standard curve shows whether a linear relation is observed in the standard curve.

2.7.3.4 Specificity of amplification products assessed by melting curve

After 45 cycles, a melting curve was generated by heating the sample to 95 °C for 60 s and then at 55 °C for 30 s. The samples were slowly heated at 0.01°C / s to 95°C and all data were collected during the ramp. Fast loss of fluorescence is observed at the denaturing/melting temperature of DNA fragment, which is a unique feature of that fragment.

The melting peak can be obtained by plotting the negative first derivative of fluorescence against temperature. Finally the samples were cooled down to 40 °C for 30 s. Melting curve analysis demonstrated that each of the primer pairs amplified a single predominant product. Each primer pair had a distinct melting temperature which was used to identify specific products in subsequent analysis.

2.7.3.5 Evaluation of RT-PCR products using agarose gel electrophoresis

As an additional control of primer specificity, the real-time PCR products were also confirmed on 1-2% agarose gels following electrophoreses (BioRad Mini Submerged DNA gel apparatus) at 80 V for 1-1.5 hours. To visualize DNA bands on agarose gels, the gels were stained with ethidium bromide (0.5 µg / ml) in the dark at room temperature for 15 min.

After washing in running water for 10 min, the gels were examined under UV light and photographed. The size of newly synthesized PCR products, according to the equation displayed on the standard curve, was estimated from the migration of the DNA size markers (1 kb markers, Roche Diagnostics) run concurrently on the gels.

2.7.3.6 Sequencing of a putative Cu-Zn SOD gene fragment

Putative Cu-Zn SOD amplicons from real-time PCR were purified using columns from the Eppendorf FastPlasmid Mini Kit (Eppendorf, Hamburg, Germany). The purified PCR products were then sequenced on an ABI3100 Genetic Analyzer using the BDT v3.1 protocol using the same primers as for real-time PCR amplification. Raw DNA sequence data were checked manually, compared with electropherograms, and further edited if necessary to improve the quality and reliability of the data, using the BioEdit program. The final sequences were BLAST searched against GenBank database (http://www.ncbi.nlm.nih.gov) and aligned with known DNA sequences of Cu-Zn SOD of a petunia hybrid to confirm the identity of the fragment obtained in this study as a Cu-Zn SOD gene fragment.

2.7.3.7 Quantification of gene expression

Gene expression was quantified based on the number of threshold cycle (Ct) at which a significant increase in the florescence signal is first detected. Expression level of the target gene in each sample was normalized by the expression level of the internal reference of 18S rRNA, represented by the ratios of gene expression to 18S rRNA expression. All samples were run in triplicates.

The relative efficiency of gene-specific and internal control primers were determined. For each sample, the levels of mRNA of interest were calculated by means of the comparative cycle threshold cycle method. The difference in the levels of mRNA of a target gene between treatment and control samples was expressed as $(1+E)^{\Delta Ct}$ or the ratio of the abundance of gene of interest relative to the expression of reference gene calculated by the formula below:

$$\text{Expression ratio} = (1+E)_{\text{target gene}}{}^{\Delta Ct}{}_{\text{target gene}} / (1+E)_{\text{internal control}}{}^{\Delta Ct}{}_{\text{internal control}}$$

Notes: ΔCt: respectively the differences of Ct values between control and treatment; E: average real-time PCR amplification efficiency.

2.8 Statistical analysis

All statistical analysis was performed using the software of Statistics version 8 (Microsoft Corporation, USA). An analysis of variance (ANOVA) was performed on various factors, including vase life, senescence percentage, fresh weight, conductivity, enzyme activity, and protein and pigment content. Percentages were transformed before analysis.

The quantification plot and melting curve data for all real-time PCR runs were analyzed using Mx3000P Real-time PCR System software (Stratagene). This software was

subsequently used to calculate and plot a linear regression line between the logarithms of the Cu-Zn SOD RNA standards and the corresponding mean threshold cycle (Ct) values. One-way ANOVA was also carried out in order to study the association between time, treatment and the levels of expression of target and reference genes in petunia petals.

When the ANOVA indicted statistical significance, comparison of means following Tukey's method was generally performed at the 95% or 99% significance level to distinguish between treatments. Asterisk (*) or different letters indicate significant differences in different parameters between different treatments. Each experiment was repeated at least twice with similar results. Results are shown as the mean value with standard errors of triplicate data values obtained in two experiments. The standard error of the mean was calculated for each sample using the spreadsheet program Microsoft Excel 2003 (Microsoft Corporation, USA).

CHAPTER 3

POST-HARVEST HANDLING OF CUT GENTIAN FLOWERS: PHYSIOLOGICAL, ULTRASTRUCTURAL AND BIOCHEMICAL INVESTIGATIONS

3.1 Introduction

The regulation of flower senescence is a central problem in plant biology. From a practical point of view, investigations into this problem are of great interest to the cut flower industry as well. A number of factors, such as pulsing solution and harvest maturity, are known to affect the post-harvest life of cut flowers. Silver thiosulfate (STS) is currently widely used as a post-harvest treatment to maintain the quality of freshly cut flowers (Bhattacharjee and De, 1998; Goszczynska and Zieslin, 1993; Guo and Sheng, 1999; Jiang et al., 1994; Serek, 1993). It has been reported that pre-treatment after harvest of cut gentian stems bearing flowers (herewith referred to as flower stems) in a solution containing STS also delayed senescence of the flowers (Zhang and Leung, 2001). However, STS and other heavy metal-based solutions as floral preservative treatments have environmental implications. The widely used STS in cut flower industry is a potential environmental hazard. An aim of this study was to explore alternative post-

harvest treatments of cut flowers that maybe more environmentally friendly. Moreover, studies were carried out to investigate how these treatments might prolong vase life of gentian flowers.

Gibberellic acid (GA) is known to regulate various aspects of plant growth and development, such as germination, cell growth, stem elongation, fruit development and pigmentation (Vainstein et al., 1994). In flowers, a decline in levels of endogenous gibberellins has been reported to be associated with the termination of flower maturation and the onset of the senescence process (Saks and Van, 1993a; Saks and Van, 1993b). Previous studies have shown that exogenous application of GA, a component of certain commercial flower preservative solutions, can delay senescence of some cut flowers (Eason et al., 2002b; Eason et al., 2004). With regard to senescence mechanisms, one major factor for flower senescence is excessive oxidative stress and / or oxidative damage of cellular macromolecules such as proteins, lipids and nucleic acids. This is correlated with generation of excessive reactive oxygen species (Bartoli et al., 1996) and / or down-regulation of non-enzymatic (carotenoid, ascorbate, glutathione, and tocopherol) and / or enzymatic (SOD, AP and POD) antioxidative defense (Kanazawa et al., 2000; Ma and Zhu, 2003; Panavas and Rubinstein, 1998a). However, relatively few studies have addressed the relationship between the anti-senescence effect of GA and the status of scavengers of reactive oxygen species. Also very little is known about the influence of exogenous GA application on the ultrastructural changes in flowers.

In this study the possible anti-senescence effect of GA_3 was investigated first in cut gentian flower stems and then confirmed later in both detached gentian flowers and isolated gentian petals. Ultrastructural changes and levels of selected antioxidants as influenced by GA_3 treatment were investigated in order to elucidate the role of GA_3 in the regulation of gentian petal senescence. Moreover, some physiological and ultrastructural changes during development and senescence of gentian petals in field-grown plants under ‘normal’ or natural cropping conditions were also studied as no such prior study is available. This serves as valuable background knowledge for the present investigations

using flowers borne on cut gentian stems as well as detached flowers and isolated half petals.

3.2 Results

3.2.1 General changes associated with flower development and senescence in gentian

Gentian flowers from GB (Stage 1), BB (Stage 3), FLO (Stage 6) and SS (Stage 8) were used for studying the general physiological changes during petal development and senescence (*Plate 2.1*). During petal development, from the green bud stage to the blue bud stage, there was a decrease in chlorophyll contents and an accompanying increase in anthocyanin content (change in absorbance of petal extracts at 530 nm) (*Figures 3.1 A* and *B*). After this, the levels of both chlorophyll and anthocyanin remained unchanged although petal weight and length increased continuously during development of blue buds to open flowers (*Figures 3.1 C* and *D*).

The first visible sign of advanced symptoms of senescence in gentian petals is that the petal tip turned brown and the rest of the blue petal showed the first sign of fading. This is one of the characteristics of Stage 7 flowers. The levels of pigments particularly the antioxidant pigment carotenoids declined early on during petal senescence (*Table 3.1*). Furthermore, an obvious increase in ovary weight (*Table 3.2*), electrolyte leakage from petals (*Figure 3.2*) and a decrease of petal cell sap pH (*Table 3.2*) at the onset of senescence from Stage 7 preceded a later decrease in petal fresh weight at Stage 8 (*Table 3.2*).

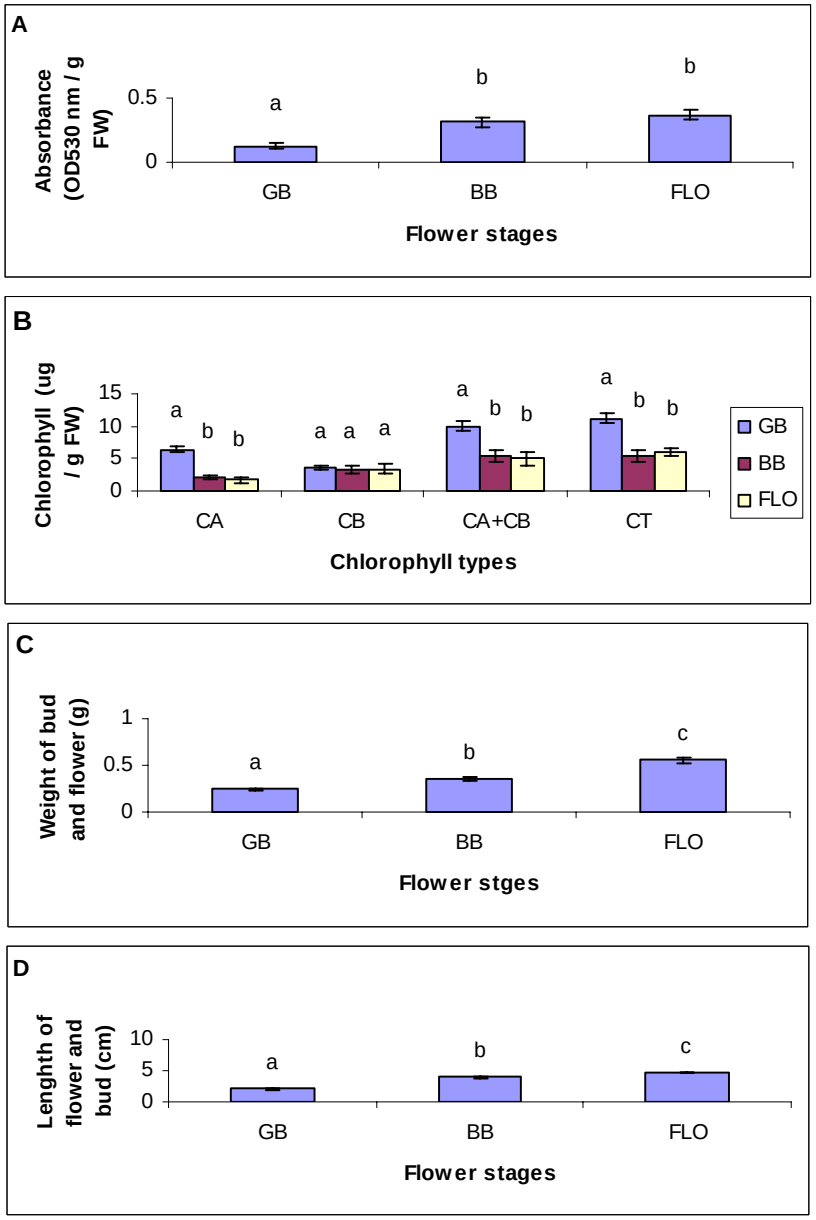


Figure 3. 1 Changes of anthocyanin (A), chlorophyll (B) levels, average bud / flower weight (C) and average bud / flower length (D) during gentian flower development

CA: chlorophyll A; CB: chlorophyll B; CT: total chlorophyll; GB: green bud; BB: blue bud; FLO: open flower. The results assigned with different letters are significantly different ($P<0.05$) using Tukey's method following ANOVA.

Table 3. 1 Changes of pigment contents in gentian petals during senescence

Pigment content	Stage 6	Stage 7	Stage 8
CA (µg / g FW)	139.17 ± 5.46 a	104.59 ± 30.05 ab	49.45 ± 13.16 b
CA (µg / petal)	28.55 ± 1.19 a	17.07 ± 5.41 ab	4.92 ± 0.72 b
CB (µg / g FW)	281.14 ± 10.70 a	217.62 ± 52.54 a	217.62 ± 14.83 a
CB (µg / petal)	57.66 ± 2.23 a	35.55 ± 10.77 a	12.26 ± 0.63 a
CT (µg / g FW)	420.31 ± 16.16 a	322.20 ± 82.59 ab	169.13 ± 28.00 b
CT (µg / petal)	86.20 ± 3.42 a	52.62 ± 16.18 ab	17.19 ± 1.35 b
AC (OD 530nm / petal)	1.33 ± 0.02 a	1.07 ± 0.10 ab	0.91 ± 0.06 b
CN (µg / g FW)	103.30 ± 3.50 a	13.69 ± 1.48 b	4.82 ± 0.40 b
CN (µg / petal)	21.18 ± 0.69 a	2.19 ± 0.35 b	0.47 ± 0.11 b

Abbreviations: CA (chlorophyll A); CB (chlorophyll B); CT (total chloropyhyll); AC (anthocyanins); CN (carotenoids). Petal senescence: Stage 6-the tip of the flowers became wide open; Stage 7-the blue color of the petal beginning to fade away; Stage 8-the petal starting to turn brown. Mean values ± SE within a row assigned with different letters are significantly different ($P<0.05$) using Tukey's method following ANOVA.

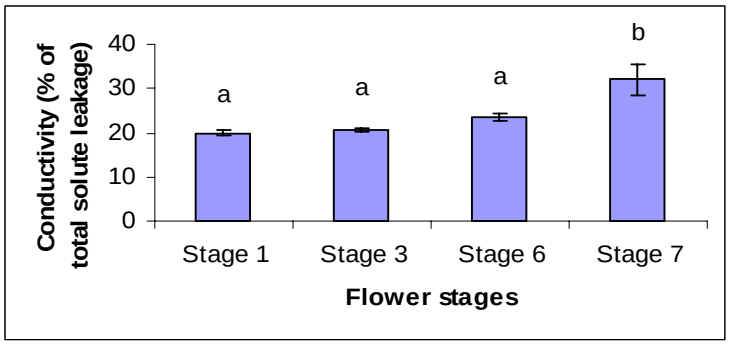


Figure 3. 2 Changes of solute leakage (%) from petals during gentian flower development and senescence

Petal development and senescence: Stage 1-green bud; Stage 3-blue bud; Stage 6-tips of the flowers became wide open; Stage 7-the blue color of the petal beginning to fade away. Vertical bars represent mean values ± SE. Results assigned with the same letter are not significantly different at P = 0.05 using Tukey's method following ANOVA.

Table 3. 2 Changes of fresh weight of petal, ovary and cell sap pH during gentian flower senescence

Items	Stage 6	Stage 7	Stage 8
Weight (g / petal)	0.62 ± 0.01 a	0.48 ± 0.03 ab	0.32 ± 0.06 b
Weight (g / ovary)	0.22 ± 0.013 a	0.50 ± 0.04 b	0.75 ± 0.05 c
Cell sap pH	5.87 ± 0.02 a	5.60 ± 0.06 b	5.27 ± 0.03 c

Petal senescence: Stage 6-tips of the flowers became wide open; Stage 7- the blue color of the petal beginning to fade away; Stage 8-blue petals become brown. Mean values ± SE within the same row followed by different letters indicate that the results are significantly different ($P<0.05$) using Tukey's method following ANOVA.

3.2.2 Ultrastructural changes during gentian petal development and senescence

Ultrastructure of gentian petals at three different developmental stages (Stage 3-blue bud, Stage 6-open flower and Stage 7-starting senescence) was studied to determine the major subcellular changes during the natural course of petal development and senescence in gentian. The following abbreviations are used in most of the following ultrastructure-related figures: cell wall (CW); rough endoplasmic reticulum (RER); chloroplast (CLP); chromoplast (CRP); plastid envelope (PE); thylakoid grana (TG); dense inclusion body (DIB); thylakoid (THY); intercellular space (IS); mitochondria (M); nucleus (N); cytoplasm (CYT); tonoplast (T); vacuole (V); Golgi body (G); Golgi vesicles (GV); ribosome (R); lipid (L); membrane vesicles (MV) plastoglobuli (PG); plasmamembrane (PM); membrane / membrane fragment (M / MF); peripheral internal membranes (PIM); starch (STA) and stroma (STR).

In the epidermal cells of blue bud (Stage 3) tissue of gentian, many chloroplasts of different sizes and structural complexities and electron-lucent mini-vacuoles were observed (*Plates 3.1 A* and *C*). At a higher magnification, the cytoplasm of these petal cells was found to be rich in mitochondria (M), densely populated with ribosomes (R) and traversed by rough endoplasmic reticulum (RER) (*Plates 3.1 B* and *D*). Well-developed Golgi bodies (G) were abundant and active in producing numerous Golgi vesicles (GV) around them (*Plate 3.1 D*).

As the blue bud expanded and developed into an open flower, numerous small vacuoles formed within petal cells during the earlier cell developmental period (*Plate 3.1 A*) were replaced by fewer large vacuoles. Later a large central electron-lucent vacuole was formed (*Plate 3.2 A*). The turgor pressure produced in the central vacuole seemed to have forced and confined the cytoplasm to a thin peripheral layer with the plasma membrane pressed tightly against the cell wall (*Plate 3.2 A*).

In the petal cells of open gentian flowers, well developed chloroplasts with a dense stroma and tightly stacked thylakoids, abundant mitochondria with many cristaes and electron-dense matrix were embedded in a ribosome-rich cytoplasmic matrix (*Plates 3.2 B, C* and *D*). Some ribosomes (R) aggregated together to form polysomes in the ground cytoplasm as well (*Plates 3.2 D*). Golgi bodies (G) and endoplasmic reticulums (ER) were also observed inside the cytoplasm of these cells *(Plates 3.2 C)*.

Various morphologically different plastids, containing plastoglobuli (darkly stained bodies), appeared in the cytoplasm of petal cells at Stage 6. They included starch granules (*Plate 3.3 A*), thin linear membrane (*Plate 3.3 B*) or a lot of membrane vesicles (*Plate 3.3 C*). These plastids were generally of spherical or oval shape and different from the spindle-formed chloroplasts. In some cells, very dense inclusion bodies appeared in plastids, and they generally occupied a large proportion of the space of those plastids (*Plate 3.3 D*). The accumulation of lipid components was a prominent feature accompanied with plastid development and maturation during gentian flower development.

In the cells at the tip of petals showing the first sign of senescence, most of the general senescence-associated ultrastructural changes such as the degradation of cell walls, membranes, cytoplasm and organelles especially plastids (chloroplast / chromoplast) were observed in gentian petals. One feature was that different spherical electron-dense membrane structures appeared in the cytoplasm at this period (*Plate 3.4 C*). Evidence was obtained for the early development of this kind of membranous structure in continuity with the tonoplast which invaginated and sequestrated part of the cytoplasmic material into it (*Plate 3.4 B*). Early formation of vesicles or membranous structures from other organelles was also shown in *Plate 3.4 D*. The rupture of the tonoplast initiated the autolysis process, which was important for degradation of other cytoplasmic components later. The integrity of plastids was lost as evident by the loss of electron density in some parts of their membrane. There was a decrease in the intensity of small plastoglobuli around the irregular remnants which were possibly those of degraded thylakoids (*Plates 3.5 A* and *B*). More advanced degradation of plastids in petal cells occurred as the

breakdown of part of the plastid membrane was accompanied with therelease of the matrix and content of the plastids (*Plates 3.5* C and *D*).

An intact cell wall was composed of tightly packed fibrils which were evenly darkly stained when observed at high magnifications (*Plates 3.4 A* and *3.5 B*). During petal senescence dissolution of the middle lamella was observed as there was a loss in electron density and apparent loosening of the cell wall striations (*Plates 3.4 B* and *3.5 A*). The dissolution of other parts of the cell wall was also observed (*Plate 3.7 B*). Cell wall buckling, one of the characteristics of cell senescence, appeared in some gentian petal cells as well *(Plate 3.6 C*).

With regard to the cytoplasm, it was progressively less electron-dense due to obvious reduction in granularity and ribosome content. Cells became devoid of contents except numerous vesicles and irregular residual membrane containing bodies aggregated in petal cells (*Plates 3.6 A* and *D*). They appeared in the cytoplasm or along the cell wall following the breakdown of plasmalemma (*Plates 3.6 B, C* and *D*).

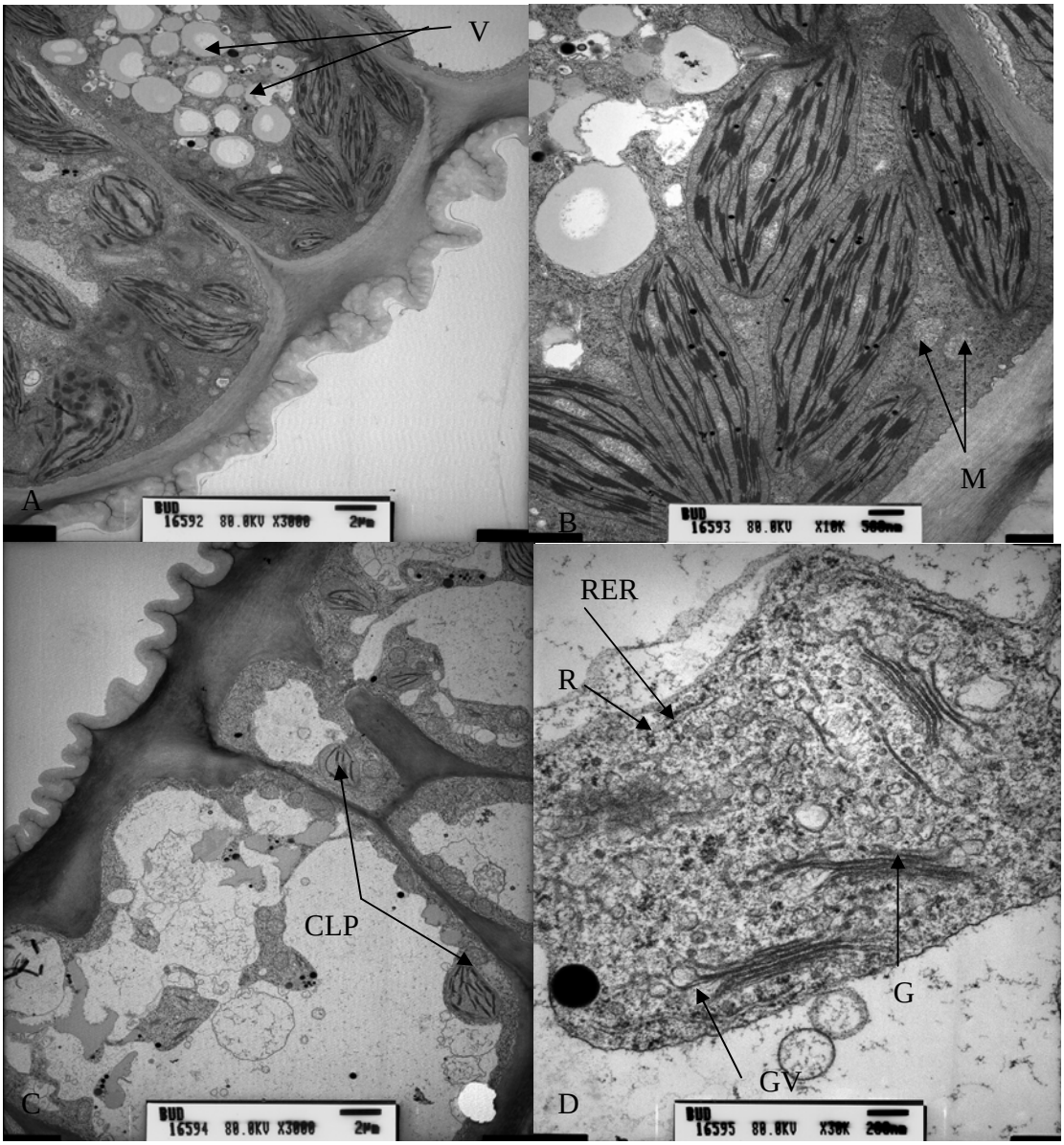


Plate 3. 1 TEM photographs of epidermal cells in blue floral bud tissue of gentian

A, B and C, epidermal cells with plentiful cytoplasmic organelles (magnifications = 3000 X, 10000 X and 3000 X, respectively); D, a cell with active Golgi bodies (mag. = 30000 X). Scale bars are A, 2 micrometers; B, 300 nanometers; C, 2 micrometers; D, 200 nanometers. CLP, chloroplast; G, Golgi body; GV, Golgi body vesicle; M, mitochondria; RER, rough endoplasmic reticulum; V, vacuole; R, ribosome.

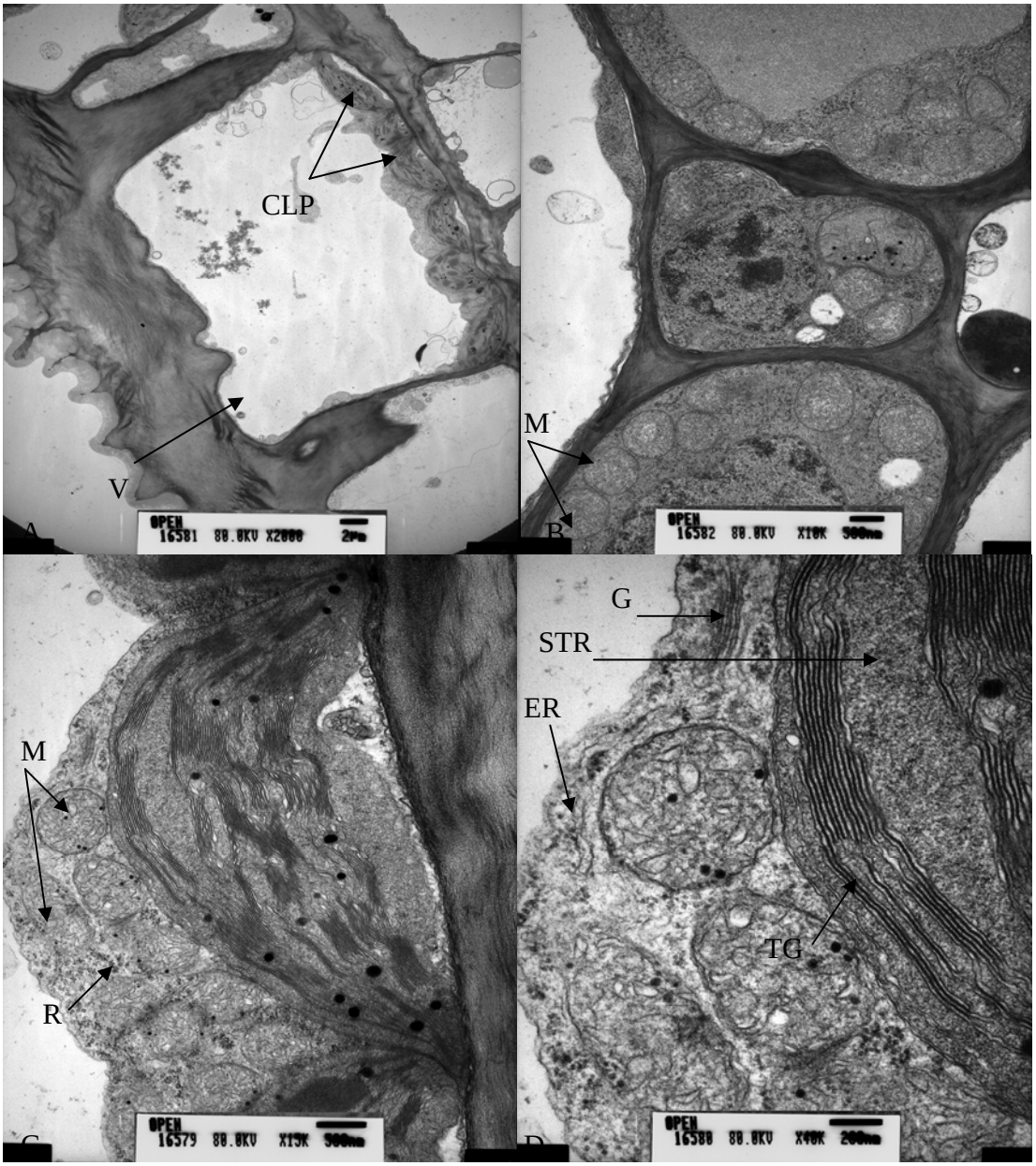


Plate 3. 2 TEM photographs of well-developed petal cells of flowers at Stage 6

A, peripheral location of cellular contents (magnification = 2000 X); B, abundant mitochondria (mag. = 10000 X); C and D, plentiful mitochondria and well developed chloroplasts (mag. = 15000 and 40000 X, respectively); Scale bars are A, 2 micrometers; B, C and D are 500, 500 and 200 nanometers, respectively. M, mitochondria; V, vacuole; G, Golgi body; TG, thylakoid grana; R, ribosome; S, stroma; ER, endoplasmic reticulum.

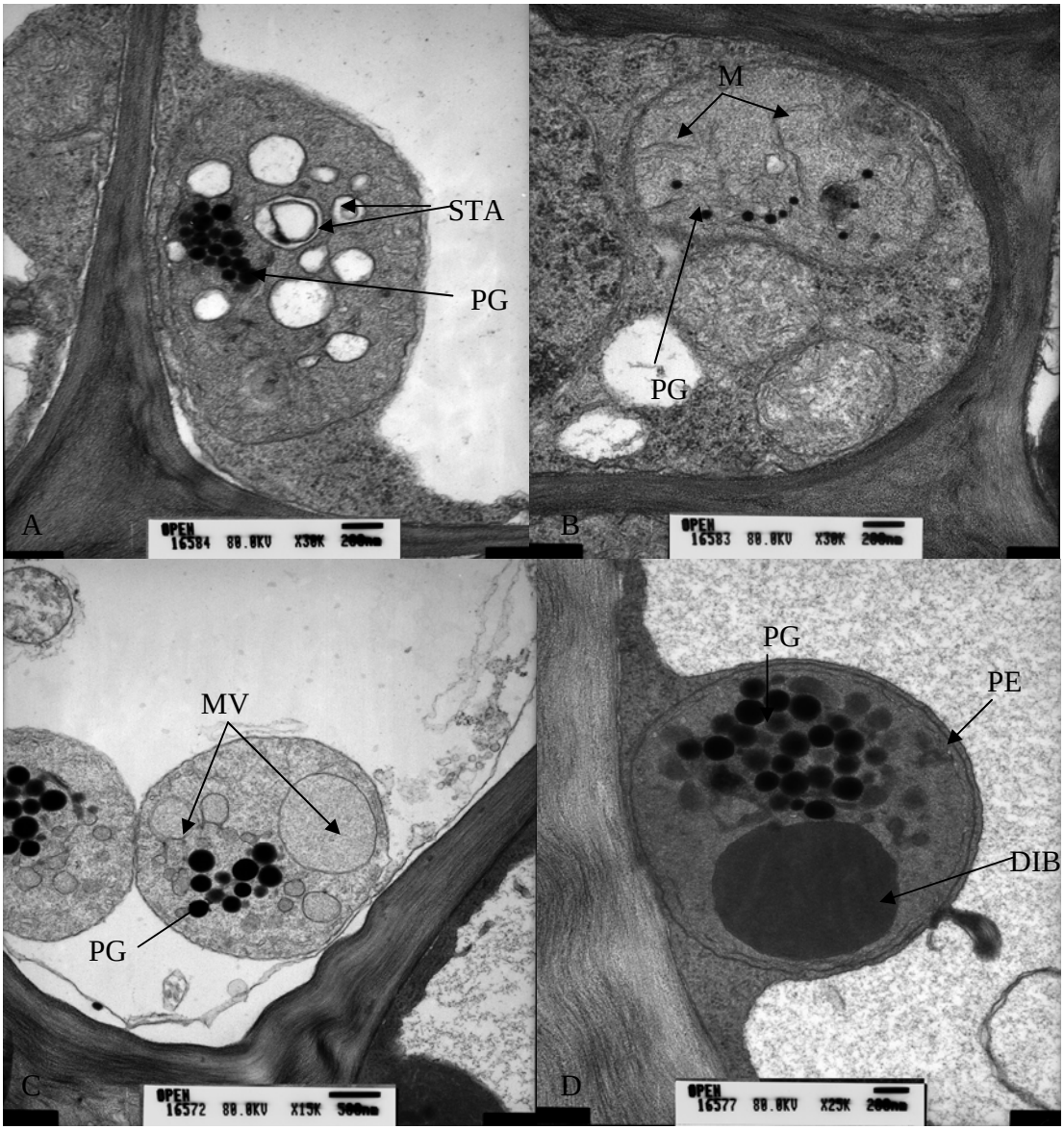


Plate 3. 3 TEM photographs of plastids within petal cells of flowers at Stage 6

A, plastid with starch granules (magnification = 30000 X); B, plastid with thinner and longer linear membranous structure (mag. = 30000 X); C, plastids with a lot of vesicles around plastoglobuli (mag. = 15000 X); D, a plastid with densely stained inclusion bodies (mag. = 25000 X). Scale bars of A, B, C and D are 200, 200, 500 and 200 nanometers, respectively. STA, starch; PG, plastoglobuli; M, membrane; MV, membrane vesicle; PE, plastid envelope; DIB, dense inclusion body.

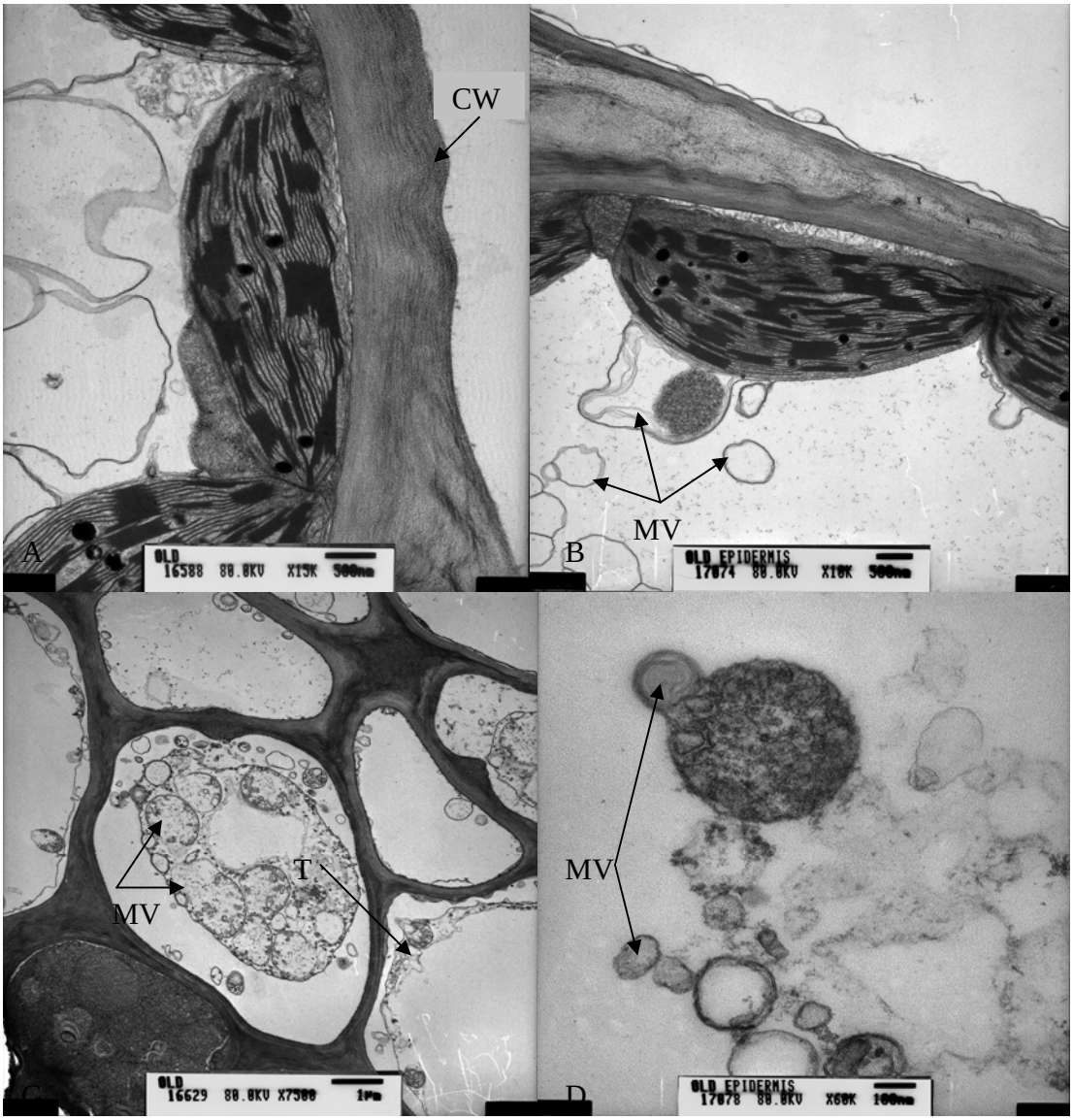


Plate 3. 4 TEM photographs of cells from the tip of a gentian petal first showing visible signs of senescence at Stage 7

A, a normal petal cell (magnification = 10000 X); B, early development of the invagination of the tonoplast including part of cytoplasic materials (mag. = 15000 X); C, a cell thought to have undergone vacuolar rupture (mag. = 10000 X); D, membrane vesicle formation from a protrusion extending from an organelle (mag. = 60000 X). Scale bars of A, B, C and D are 500, 500, 500 and 100 nanometers, respectively. MV, membrane vesicle; CW, cell wall; T, tonoplast.

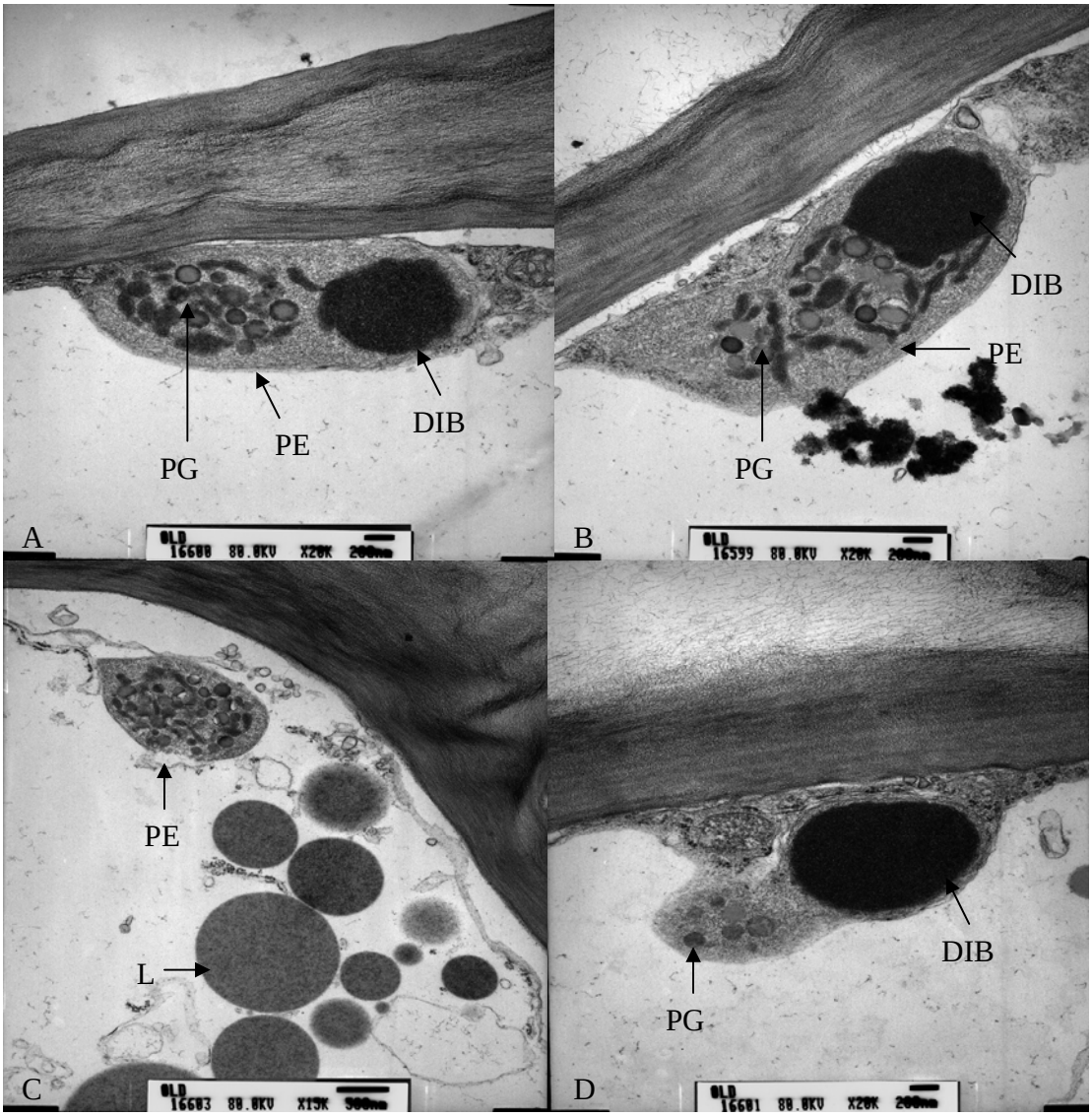


Plate 3. 5 TEM photographs of cells with different stages of plastid degradation during gentian petal senescence

A, Changes in the middle lamella of cell wall and the loss of electron density of plastid membrane (magnification = 20000 X); B, degradation of plastoglobuli (mag. = 15000 X); C, breakdown of plastid membrane (mag. = 20000 X); D, degradation of plastid membrane concomitant with release of its content (mag. = 20000 X). Scale bars of A, B, C and D are 200, 500, 200 and 200 nanometers, respectively. L, lipid; PG, plastoglobuli; PE, plastid envelope; DIB, dense inclusion body.

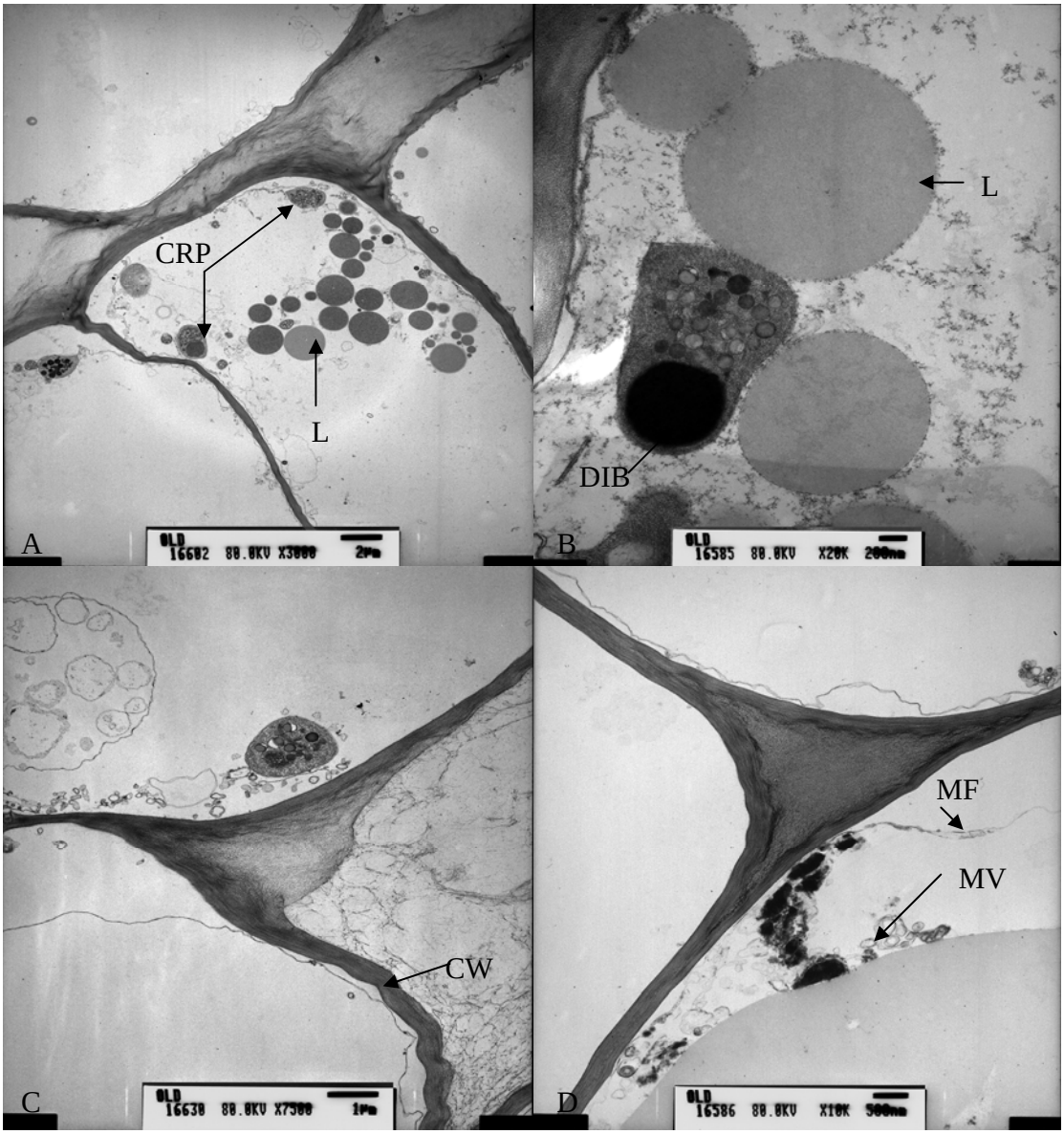


Plate 3. 6 TEM photographs of cell degradation at a late stage of senescence of gentian petal tissues

A, lipid droplets dispersed in the cytoplasmic sap (magnification = 3000 X). B, another specimen showing lipid droplets close to a plastid (mag. = 20000 X); C and D, residual membranous structures following cytolysis (mag = 7500 X and 10000 X, respectively). Scale bars are A, 1 micrometer; B, 500 nanometers; C, 200 nanometers; D, 2 micrometers. CRP, chromoplast; L, lipid; MF, membrane fragment; MV, membrane vesicle; CW, cell wall; DIB, dense inclusion body.

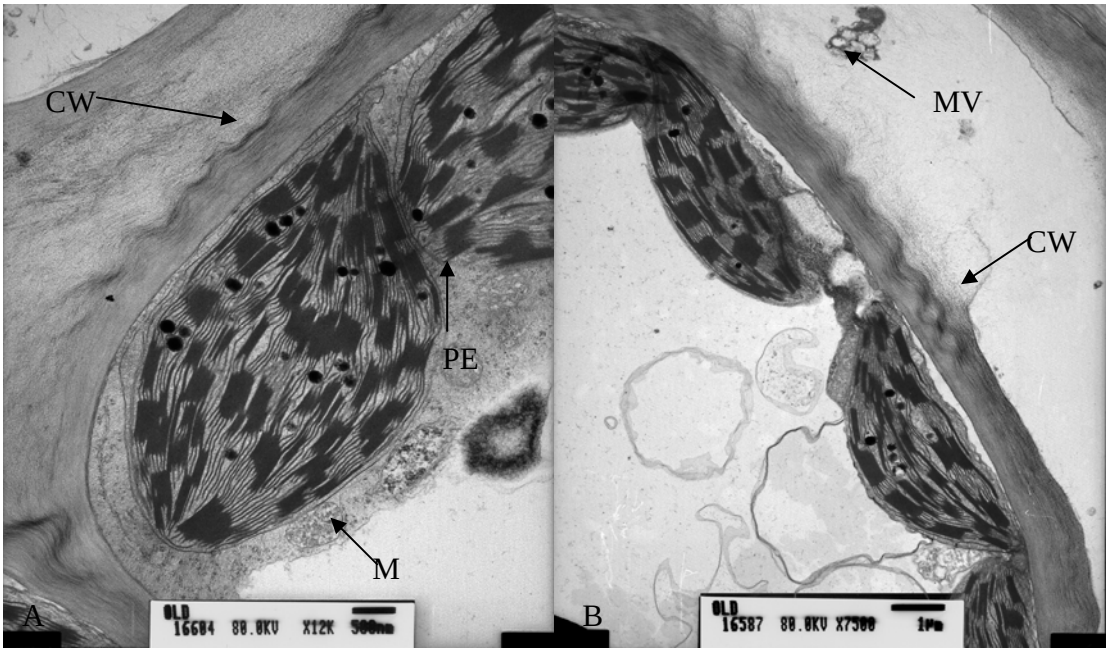


Plate 3. 7 TEM photographs of cells at a late stage of senescence of gentian petal tissues

A, the loss of electron density of cell wall (magnification = 12000 X); B, dissolution of cell wall (mag. = 7500 X). Scale bars are A, 500 nanometers; B, 1 micrometers. MV, membrane vesicle; CW, cell wall; PE, plastid envelope; M, mitochondrion.

3.2.3 Effect of post-harvest treatments on senescence of flowers borne on cut gentian stems

3.2.3.1 Screening for anti-senescence chemicals

In a preliminary trial, cut gentian stems bearing flowers were pulsed with one of the following solutions: distilled water (the control), 5 μM GA_3, 0.5 mM spermine, 0.3 μM kinetin for 24 hours. In addition, some were also treated with cycloheximide (0.1 mg/L) for 12 hours. Following the various treatments, all stems were placed in distilled water containing 200 mg/L 8-HQS. Then the vase life of these flower stems was assessed daily.

On day 14 following the pulsing treatments, 54.94%, 13.64 % and 30.65% of the gentian flowers in the control, GA_3- and spermine-treated stems, respectively exhibited visible symptoms of senescence (*Figure 3.3*). Another week later, more flowers exhibited signs of senescence in the kinetin and cycloheximide pulsing treatments compared to the control. The anti-senescence effect of GA_3 in gentian will be examined in detail in later experiments.

3.2.3.2 Effect of sucrose and GA_3 on the opening and senescence of flowers on cut gentian stems

In a subsequent trial, stems were harvested at a less mature stage (with several flowers on the stems almost open fully). They were treated with different concentrations of GA_3 with and without sucrose added. Some differences among these pulsing treatments were found in terms of the time (days) taken for the first flower to show visible symptoms and 50% of the flowers that had shown symptoms of senescence on a cut gentian stem. In the water control, the first florets were open about 2 days after pulsing the stems. The first sign of flower senescence became visible at about day 20 (*Table 3.3*). Pulsing with different concentrations of GA_3 or sucrose had no significant effect on the time of the first flower to open, but influenced the time for the first flower to show symptoms of senescence. Application of sucrose with or without GA_3 also extended the time for the first flower to become senescent on gentian stems to about 27 days compared to about 20 days in the control.

A closer examination of the effects of different concentrations of GA_3 (*Figure 3.4 A*) and sucrose (*Figure 3.4 B*) revealed that both GA_3 and sucrose affected the process of gentian flower senescence. Among the different concentrations of GA_3 tested, 5 µM was used to test the interaction of GA_3 with 3% sucrose on gentian flower senescence as well. The vase life of the flowers on the stems (as indicated when 50% of the flowers had senesced) was significantly extended by the pulsing treatments of a mixture of GA_3 and sucrose as well (*Table 3.3*).

With regard to flower opening, in gentian the majority of flowers on the cut stems was open before the initiation of flower senescence. On day 16 more than 80% of the flowers per stem had opened (*Figure 3.5 A*) while less than 7% of the flowers had senesced (*Figure 3.4 A*). Both GA_3 and sucrose could slightly speed up the process of gentian flower opening (*Figure 3.5B*).

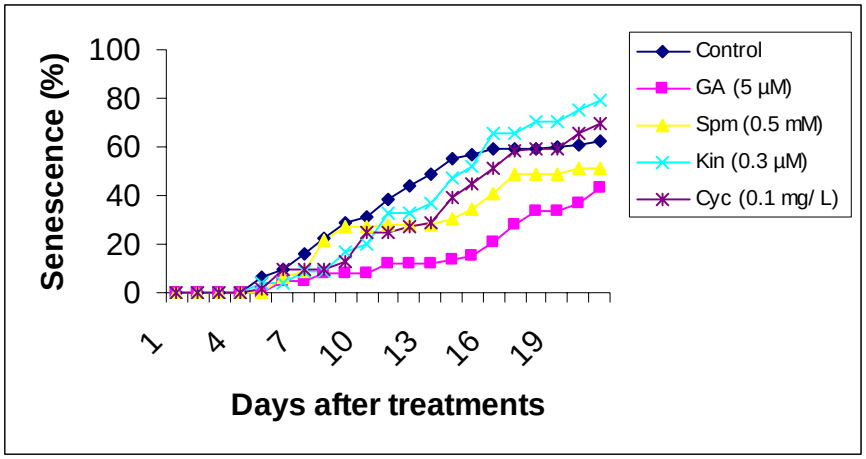


Figure 3. 3 Effect of pulsing cut gentian flower stems with different post-harvest treatments on the percentage of flowers with visible senescence symptoms

Cut gentian stems were pulsed for 24 or 12 hours with different test solutions, before they were transferred to distilled water containing 200 mg/L HQS for the entire experimental period in a growth room kept at 23º C. These experimental conditions after pulsing treatments were the same throughout this project unless indicated otherwise. Values are means of 10 replications ± SE.

Table 3. 3 Effect of pulsing cut gentian stems for 24 hours with various concentrations of GA_3 and sucrose on the time for first flower to open, become senescent and when 50% of the flowers had senesced

Treatments	1st flower to open (days)	1st flower to show senescence (days)	50% flowers became senescent (days)
Control	1.83 a	19.50 b	24.80 b
GA_3 (1000 µM)	1.67 a	22.17ab	27.00 ab
GA_3 (100 µM)	1.60 a	22.80 ab	26.25 ab
GA_3 (10 µM)	1.83 a	23.40 a	28.17 ab
GA_3 (5 µM)	1.83 a	24.60 a	27.25 ab
GA_3 (1 µM)	2.00 a	24.20 a	27.00 ab
Sucrose (3%)	2.00 a	26.83 a	28.60 ab
GA_3 (5 µM) + (3%) sucrose	1.83 a	27.67 a	30.33 a

Values are means of 5 replications ± SE. Different letters on the same particular parameter in a particular column of data indicate that the results are statistically different at P = 0.05.

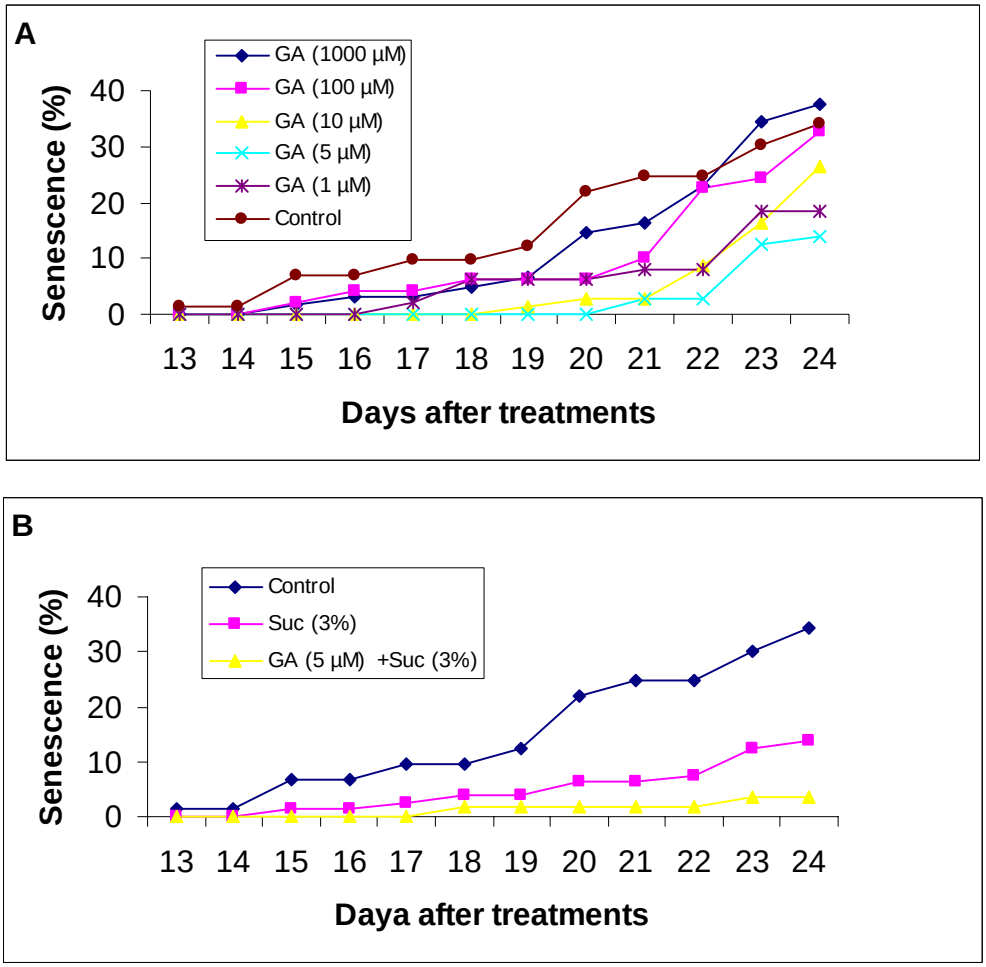


Figure 3. 4 Effect of pulsing cut gentian stems for 24 hours with various concentrations of GA_3 and sucrose on the percentage of flowers that had senesced

A, different concentrations of GA_3

B, sucrose (Suc) (3%) and GA_3 (5 µM)

Each treatment consisted of 9 stems. Assessment began on the day after the pulsing treatments.

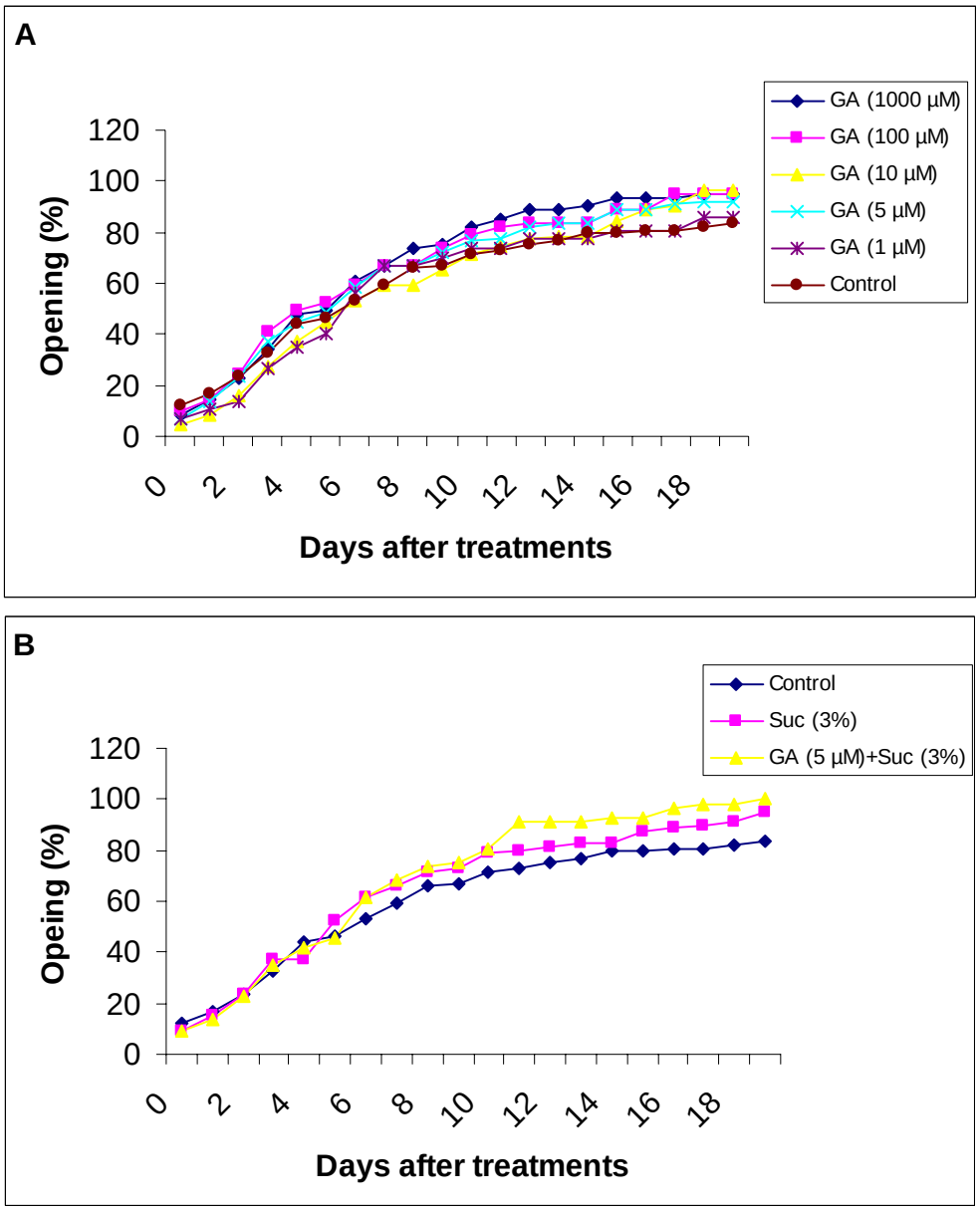


Figure 3. 5 Effects of various concentrations of GA_3 and sucrose pulsing of cut gentian stems on the percentage of gentian flowers that had become open

A: effect of different concentrations of GA_3; B: effect of sucrose (3%) and GA_3 (5 µM). Each treatment consisted of 9 stems. Assessment began on the day after the pulsing treatments.

3.2.3.3 Effect of GA_3 on changes in FW of flowers on cut gentian stems

More mature gentian stems were selected for use in this experiment. After three weeks from the pulsing treatments, the fresh weight of GA_3-treated flowers was greater than that of flowers in water control (*Table 3.4*). The anti-senescence effect of GA_3 on the flowers of cut gentian stems was associated with a retardation of flower and petal fresh weight loss. This was reconfirmed in another experiment. After pulsing with GA_3 (5 µM) and water for 32 hours, cut gentian flower stems were kept in water at room temperature. The flowers opened on a particular day (after 1, 2, 4, or 8 days from the pulsing treatments) were labeled and collected for fresh weight measurements at the end of experiment (3 weeks). From whole flower and petal weight differences, GA_3 pulsing also retained the fresh weigh of flower or petal better than water treatment alone (*Figure 3.6*). This effect was particularly noticeable in flowers that became open on day 1 or day 2 after the GA_3 pulsing treatment.

Table 3. 4 Effect of GA_3 (5 µM) pulsing of cut gentian stems for 24 hours on the fresh weight changes (g per flower or petal) of open and unopen flowers

Items	Fresh weight (g)	GA_3 application	Control
Open flowers on the stems	Flower	0.65 ± 0.06 a	0.20 ± 0.02 b
	Petal	0.45 ± 0.03 a	0.14 ± 0.02 a
Unopen flowers on the stems	Flower	0.76 ± 0.06 a	0.34 ± 0.01 b
	Petal	0.59 ± 0.03 a	0.25 ± 0.02 b

Cut gentian stems were pulsed 24 hours with 5 µM GA_3 or water (control) first and then transferred to distilled water containing 200 mg/L 8-HQS. The cut gentian stems were kept at 23º C in a growth room. The fresh weight of the whole flowers or the petals alone was determined. Data in a row assigned with different letters are significantly different ($P<0.05$). Values are means of 5 replications ± SE.

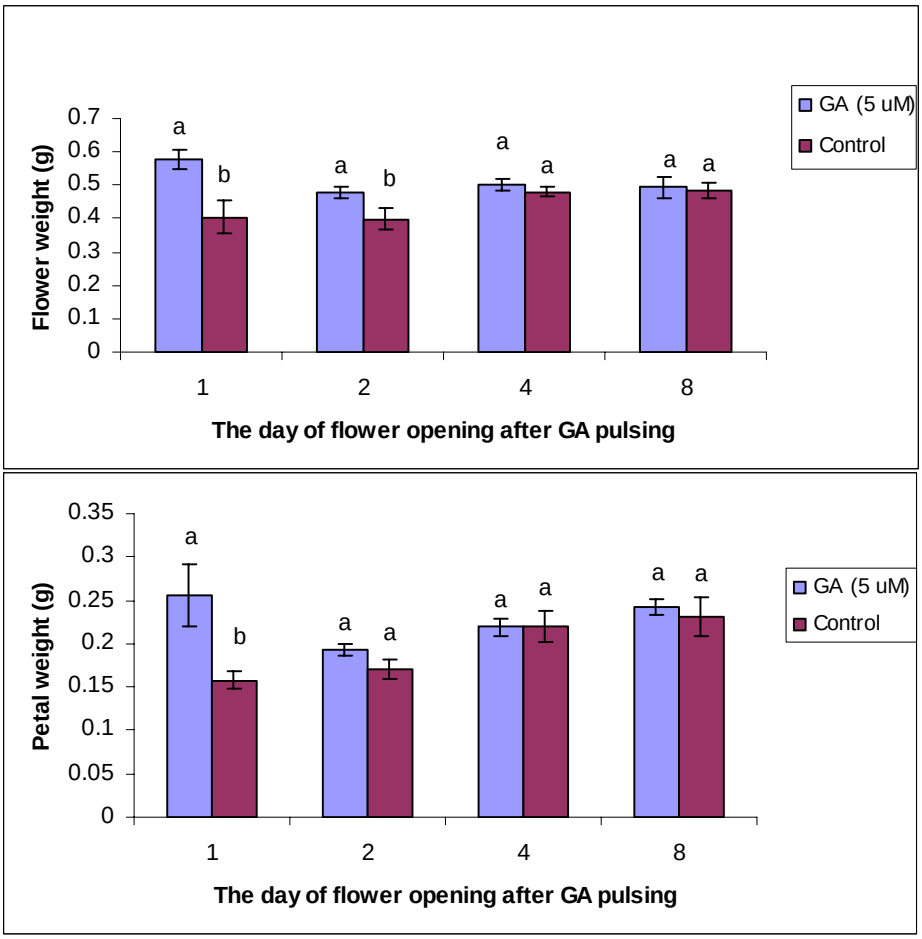


Figure 3. 6 Differences in the fresh weight of petals (the lower graph) and whole flowers (the upper graph) which opened on day 1, 2, 4 or 8 after 32 hours of pulsing with GA_3 (5 µM) or water

The stems had been kept at room temperature for three weeks. Results are means of 3 replicates ± SE. Mean values are compared using one way ANOVA. Significant differences between the two treatments comparing flowers that became open on a particular day are indicated as a and b.

3.2.4 Anti-senescence effect of GA_3 on detached gentian flowers and half petals

3.2.4.1 Effect of GA_3 on delaying petal color fading and senescence in detached gentian flowers

Thus far the results obtained showed that GA_3 could delay senescence of flowers on cut gentian stems. To see if GA_3 could act directly on the flowers rather than indirectly through the leaves and stem tissues, a detached single flower system was used. Half petal system was considered further to eliminate the possible errors from flower selection during the experiments. The results obtained revealed that GA_3 (5 μM) was also effective in both the detached single flower (*Plate 3.8*) and half petal systems (*Plate 3.9*).

In the half petal system, the first morphological change associated with senescence was that the bright blue petals slowly faded to be dark blue. Later on the petals turned brown at the top part and gradually the entire petals became brown. Finally some tissue totally lost colour and became dry. In preliminary experiments, it was found that half gentian petals could live longer after GA_3 pulsing. When the water-treated half petals (on the right side of every pair of bottles) were brown in some parts, the other GA_3-treated half petals (on the left side) were still in a relatively healthy condition (*Plate 3.9*, the lower plate). Subsequent experiments confirmed that GA_3 could delay the changes of blue color of petals (*Plate 3.9*, the upper plate) and green color of the lower vein tissue (*Plate 3.9*, the middle plate). The GA_3 (5 μM) treatment significantly delayed petal discoloration and petal senescence in half petal system (*Figure 3.7*). Similar results were also obtained in the detached single flower system (*Table 3.5*).

Plate 3. 8 Effect of different GA_3 treatments on senescence of detached gentian flowers

Upper plate: open gentian flowers; Lower plate: unopen gentian flowers. The flowers in a row were treated with a different concentration of GA_3. In every pair of bottles in a row, the right was a GA_3-treated flower; the left was water control. The flowers were pulsed in GA_3 or water for 24 hours and then transferred to distilled water containing 200 mg/L HQS and kept in a growth room. The photos were taken after 3 weeks from the start of the pulsing treatments.

Plate 3. 9 Effect of GA_3 on senescence of half gentian petals

Upper plate: the outside view of a petal from field-grown gentian plants; Middle plate: the same half petal as above, but the inside petal view; Lower plate: petals from *in-vitro* grown gentian plants. Every pair of bottles stands for one treatment and the left: GA_3-treated half of the petal; the right: water-treated half of the petal. After 24 hours pulsing with 5 µM GA_3 (G) or water (W), the petals were transferred to distilled water containing 200 mg/L HQS and kept in a growth room. The photos were taken after two weeks (upper and middle plates) and one month later (lower plate) from the start of the pulsing treatments.

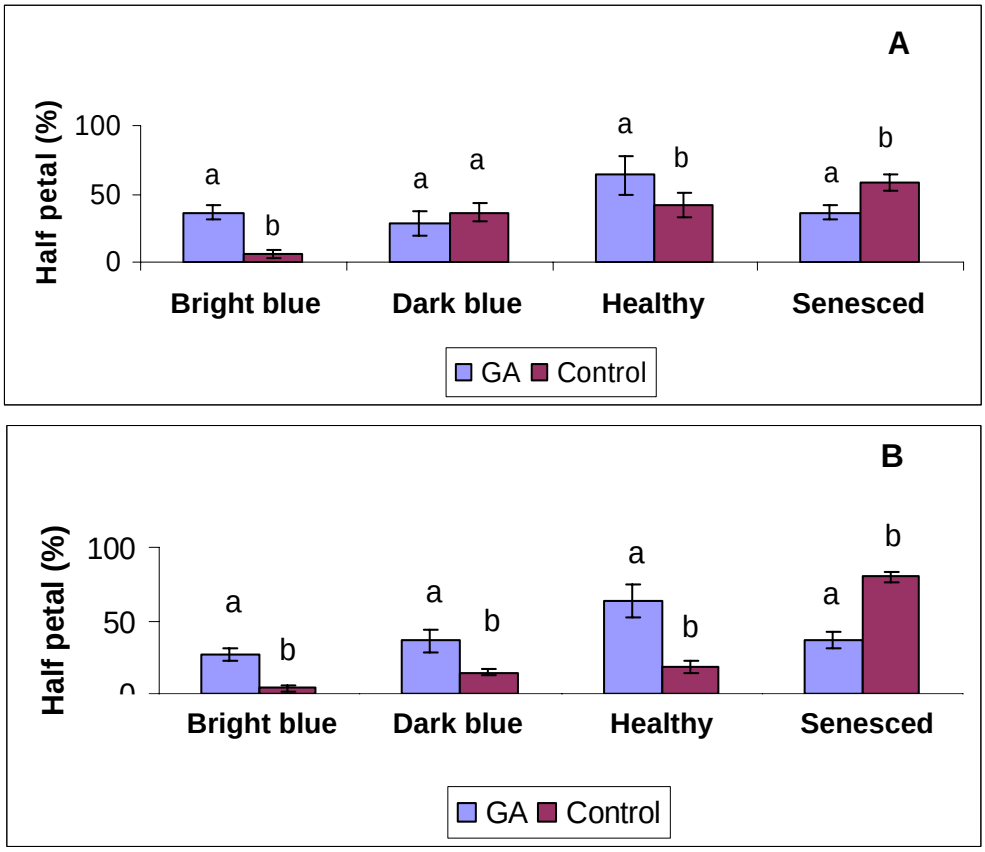


Figure 3. 7 Effect of GA_3 treatment on the colour change and senescence of isolated half gentian petals with time (n = 18)

Petals were held in water or 5 µM GA_3 for 24 hours before they were transferred to distilled water containing 200 mg/L HQS and kept in a growth room for the appropriate duration of the experiment (A: 15 days; B: 20 days). A pair of histographs with different letters indicates that the results are statistically different at P = 0.05.

The petals in a treatment were scored in two ways: firstly, the % of the petals that were bright blue and % of those with their fresh blue colour beginning to fade away. Secondly, the petals in a treatment were classified as healthy ones without any sign of turning brown or as old ones if parts of the petal tissues had become brown or yellow in the course of a treatment.

Table 3. 5 Effect of pulsing with GA_3 for 24 hours on colour change and senescence of detached single gentian flowers with time (n = 9)

Days	Treatment	(%) Bright blue	(%) Healthy	(%) Senesced
10	Control	25.93 a	55.56 a	44.44 a
	GA_3 (5 µM)	48.15 b	85.19 b	14.81 b
15	Control	3.70 a	12.04 a	87.96 a
	GA_3 (5 µM)	3.70 a	45.37 b	54.63 b

After pulsing with water or 5 µM GA_3, flowers were transferred to and held in distilled water containing 200 mg/L HQS for the duration of the experiment indicated.

The petals in a treatment were scored in two ways: firstly, the % of the petals that were bright blue and % of those with their fresh blue colour beginning to fade away. Secondly, the petals in a treatment were classified as healthy ones without any sign of turning brown or as old ones if parts of the petal tissues had become brown or yellow in the course of a treatment. Different letters on the same particular parameter indicate that the results are statistically different at P = 0.05.

3.2.4.2 Effect of GA_3 on the changes of petal weight and electrolyte leakage in detached gentian flowers

Experiments with the detached single gentian flower system showed that the loss of petal weight was significantly retarded by the 5 or 10 µM GA_3 treatment of the flowers at the unopen bud stage (*Table 3.6 A*). However, GA_3 had no effect on the open flowers. This result was consistent with that obtained with the flowers on cut gentian stems (*Table 3.4*). A decrease in petal solute leakage of only the unopen flowers was also prevented by the 5 or 10 µM GA_3 treatment (*Table 3.6 B*). Similar effects of GA_3 on petal weight (*Figure 3.8,* upper figure*)* and electrolyte leakage (*Figure 3.8,* lower figure) were observed in the isolated half petal system.

Table 3. 6 Effect of GA_3 treatment on fresh weight (A) and petal electrolyte leakage (B) of detached gentian flowers at open, unopen or bud stages

A	**Petal fresh weight (g / petal)**	
Treatments	Open flower	Unopen flower
GA_3 (1 µM)	0.18 ± 0.01 a	0.17 ± 0.01 a
Control	0.16 ± 0.01 a	0.14 ± 0.01 a
GA_3 (5 µM)	0.17 ± 0.01 a	0.17 ± 0.01 a
Control	0.14 ± 0.01 a	0.12 ± 0.01 b
GA_3 (10 µM)	0.17 ± 0.02 a	0.18 ± 0.01 a
Control	0.15 ± 0.02 a	0.14 ± 0.01 b

B	**Petal electrolyte leakage (% of total solute leakage)**	
Treatments	Open flower	Unopen flower
GA_3 (1 µM)	18.87 ± 2.579 a	24.94 ± 2.06 a
Control	23.13 ± 1.905 a	29.22 ± 3.84 a
GA_3 (5 µM)	23.02 ± 1.89 a	15.84 ± 1.38 a
Control	28.38 ± 2.19 a	24.76 ± 2.11 b
GA_3 (10 µM)	18.87 ± 1.91 a	16.27 ± 0.20 a
Control	22.20 ± 1.06 a	25.93 ± 2.15 b

Flowers were held in distilled water or 5 µM GA_3 for three weeks. Values are means ± SE (n = 3). Different letters beside values in the same column indicate that the results are statistically different at P = 0.05.

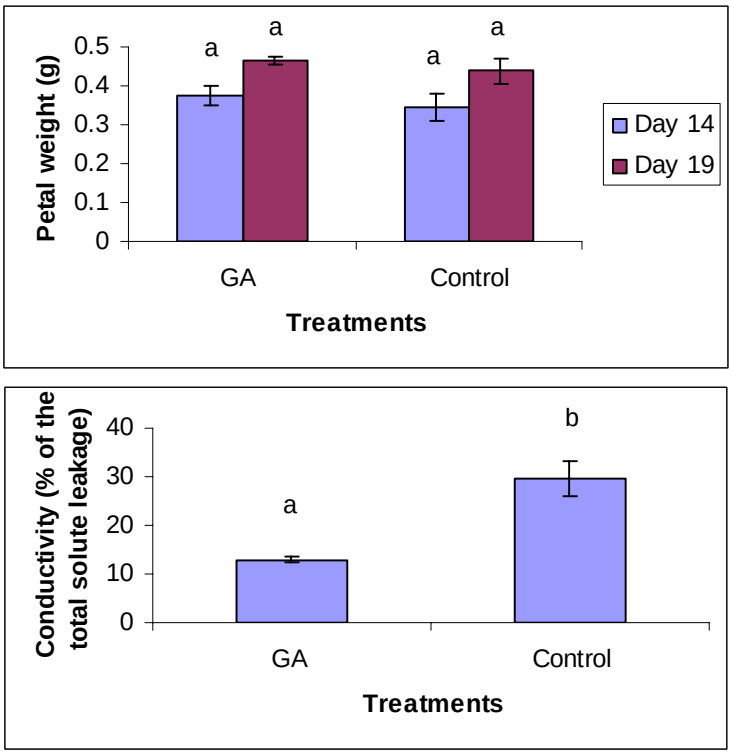


Figure 3. 8 Effect of GA_3 treatment on fresh weight changes (upper figure) and solute leakage (lower figure) of isolated half gentian petals

Petal fresh weight (g per petal) was determined after 14 and 19 days from the start of the experiment. Petal solute leakage was determined after three weeks from the start of the experiment.

Vertical bars represent means ± SE for 3 determinations from individual extracts. Different letters indicate that the results are significantly different ($p<0.05$) using Tukey's method following ANOVA.

3.2.4.3 Ultrastructural changes after GA_3 application

In order to accurately compare the effect of GA_3 on ultrastructural changes, petals were cut into two halves along the middle and pulsed separately with GA_3 or water for 24 hours. Then they were transferred to distilled water containing 200 mg/L HQS. Two weeks later, ultrastructural changes of the half petals were observed. It was found that GA_3 could modulate the development of plastids in gentian petals.

In the cells at the top part of the petal, more carotenoid-containing chromoplasts were promoted after GA_3 application. Chromoplasts of different sizes occurred more frequently in GA_3-treated gentian petals when compared to control petals (*Plates 3.10*). In some cells, much of the space of a mature plastid was filled with a great number of osmiophylic globules in GA_3-treated petals cells (*Plate 3.10 D*). Electron-dense plastoglobuli were also found in chloroplasts of the control petal cells. In this case, the appearance of plastoglobuli was presumed to be related to the degeneration of chloroplast / thylakoid system since plastoglobuli appeared at the place of degraded thylakoids (*Plate 3.10 C*; *Plates 3.11 C* and *D*; *Plate 3.14 C*).

A peripheral internal membrane system corresponding to the chromoplast internal structures (CIMs) described by Bonora et al. (2000) was also observed along the envelope of chromoplasts in gentian petals following sucrose treatment *(Plate 3.12)*. This phenomenon of CIMs was also randomly observed in plastids of GA_3-treated gentian petals (*Plates 3.10 A* and *C, plate 3.13 B*). Furthermore, some specific membrane structures (*Plates 3.13 A* and *B*) and some irregular membrane vesicles (*Plates 3.13 C* and *D*) frequently appeared in the plastids of GA_3-treated gentian petals. However these characteristics rarely occurred in control gentian petals. Generally, normal chloroplast structures were the characteristic features of most of the plastids in the cells at the top part of the petals treated with water. They showed typical well-developed chloroplast ultrastructures with several grannal thylakoid discs which were interconnected by stromal

thylakoids (*Plate 3.14 A*). In control petals chloroplasts showed different degrees of cellular degradation (*Plates 3.11 A* and *B*), particularly thylakoid degradation (*Plates 3.14 B, C,* and *D*). The degradation of thylakoids in chloroplasts was frequently accompanied by the formation of plastoglobuli (*Plates 3.11 C* and *D*).

In the cells of vein tissue at the lower part of gentian petals, it was found that senescence-associated features such as the general degradation of cell membranes, cytoplasm and organelles, particularly chloroplasts, were retarded by GA_3 treatment. When plasmalemma was partially withdrawn from the cell wall, less electron-dense cytoplasm as observed in water-treated gentian petals (*Plates 3.15 A* and *B*) when compared to GA_3-treated ones (*Plates 3.15 C* and *D*). This is possibly due to the earlier rupture of vacuoles that might have led to the advanced cytoplasmic degradation. A possible rupture of cell wall in water-treated petals was also manifested by the empty regions found inside the cell wall. Also cytoplasmic remnants were found in the intercellular spaces (*Plate 3.15 B*). The more advanced degradation of thylakoid membrane and chloroplast envelope was observed in water-treated gentian petals (*Plates 3.16 A* and *B*) when compared to the GA_3-treated ones (*Plates 3.16 C* and *D*).

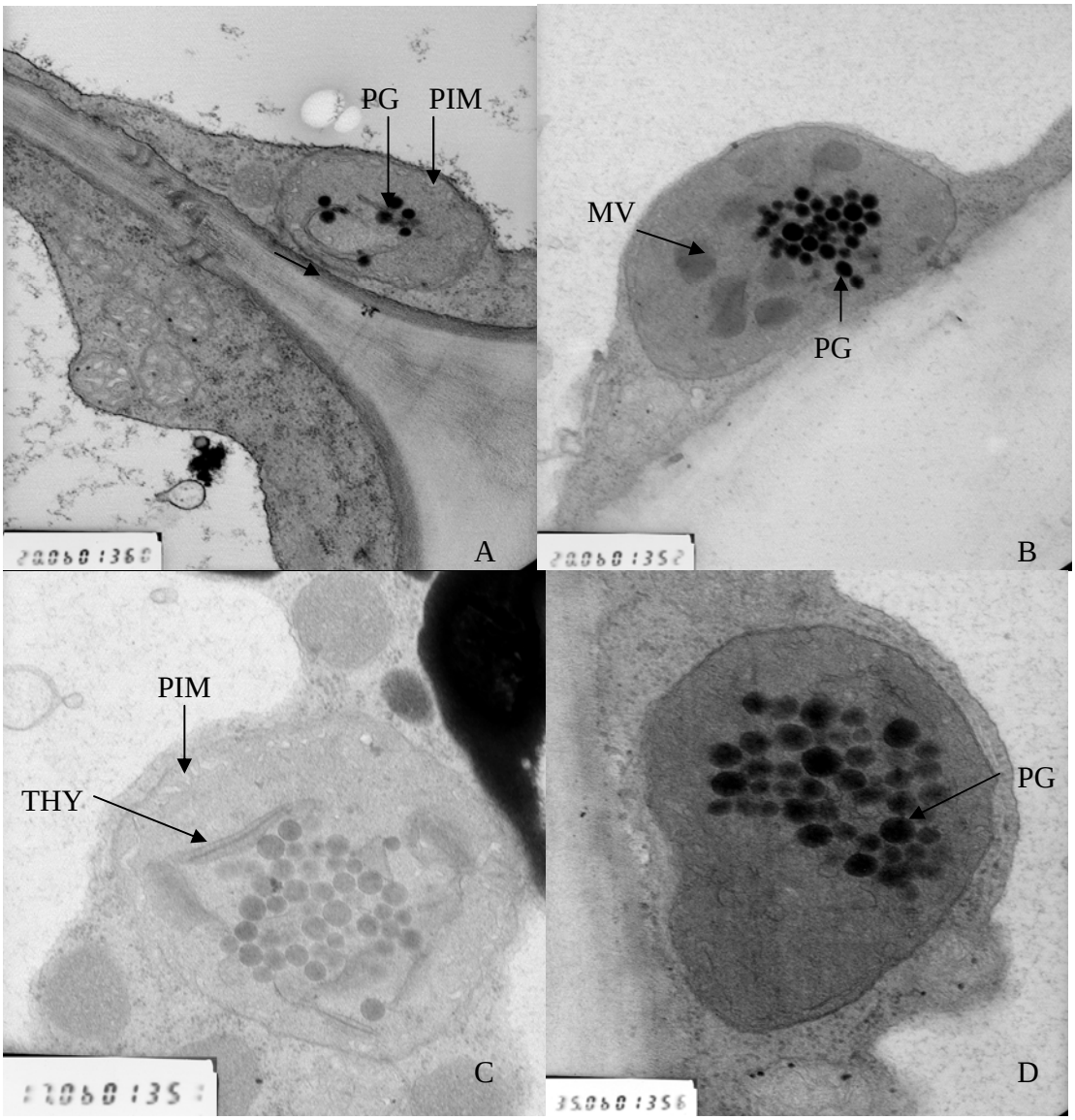


Plate 3. 10 TEM photographs of cells from the upper part of GA_3-treated gentian petals showing chromoplasts with carotenoid-containing plastoglobuli

A, plastoglobuli formed around several long perforated membranes and visible peripheral PIMs around the envelope of plastids (magnification = 20000 X); B, plastoglobuli formed around the irregular membrane vesicles (mag. = 20000 X); C, remnants of the thylakoid system accompanied by plastoglobuli (mag. = 17000 X); D, a mature chromoplast filled with numerous plastoglobuli (mag. = 35000 X). PG, plastoglobuli; PIM, peripheral internal membranes; MV, membrane vesicle; THY, thylakoid.

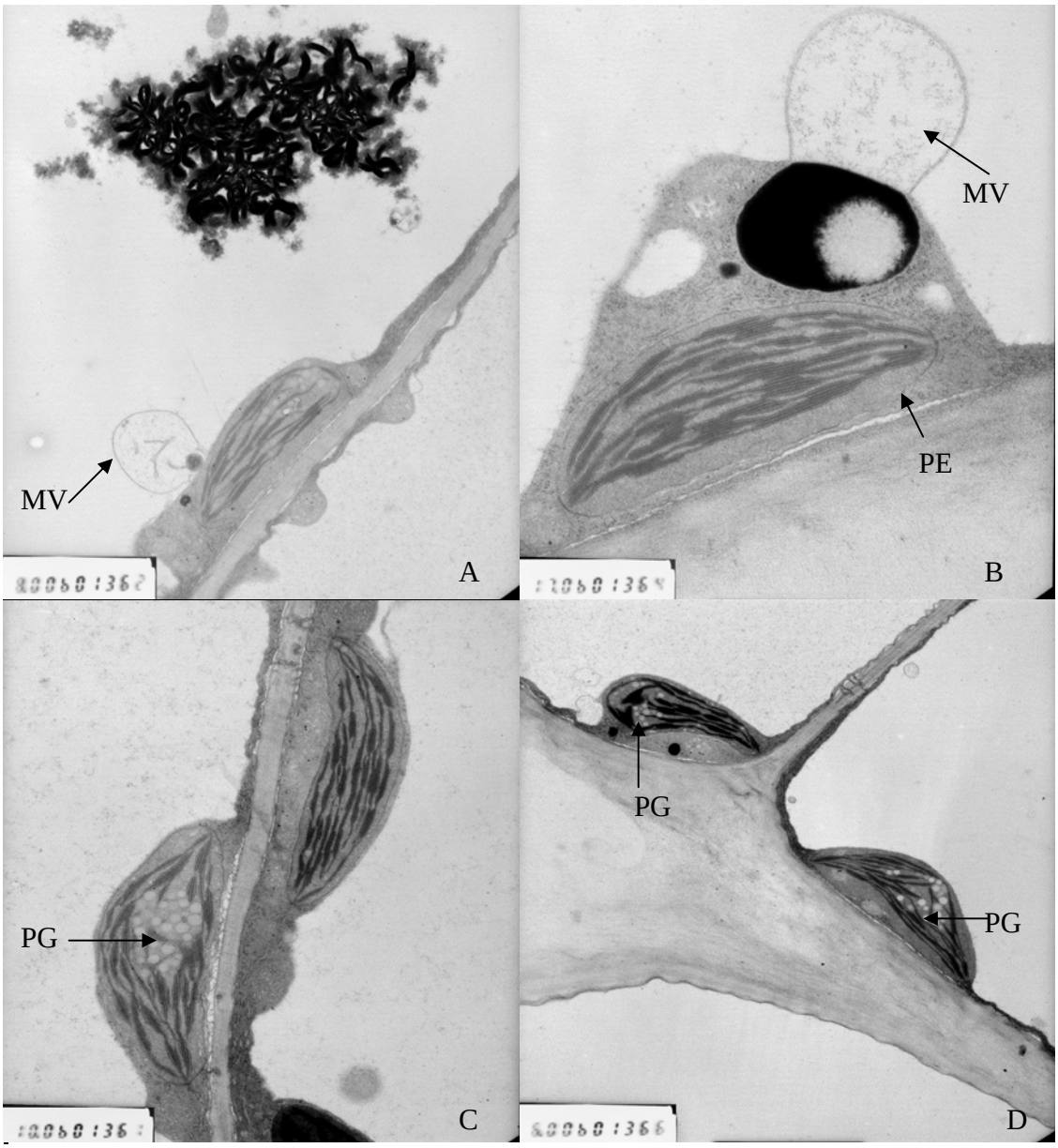


Plate 3. 11 TEM photographs of cells from the other half petal of the same gentian flower used in *Plate 3.10*. The half petal was treated with water.

A and B, cellular degradation and vesicle formation (magnifications = 80000 X and 17000 X, respectively); C and D, thylakoid degradation associated with plastoglobuli formation (mag. = 10000 X and 60000 X, respectively). PG, plastoglobuli; PE, plastid envelope; MV, membrane vesicle.

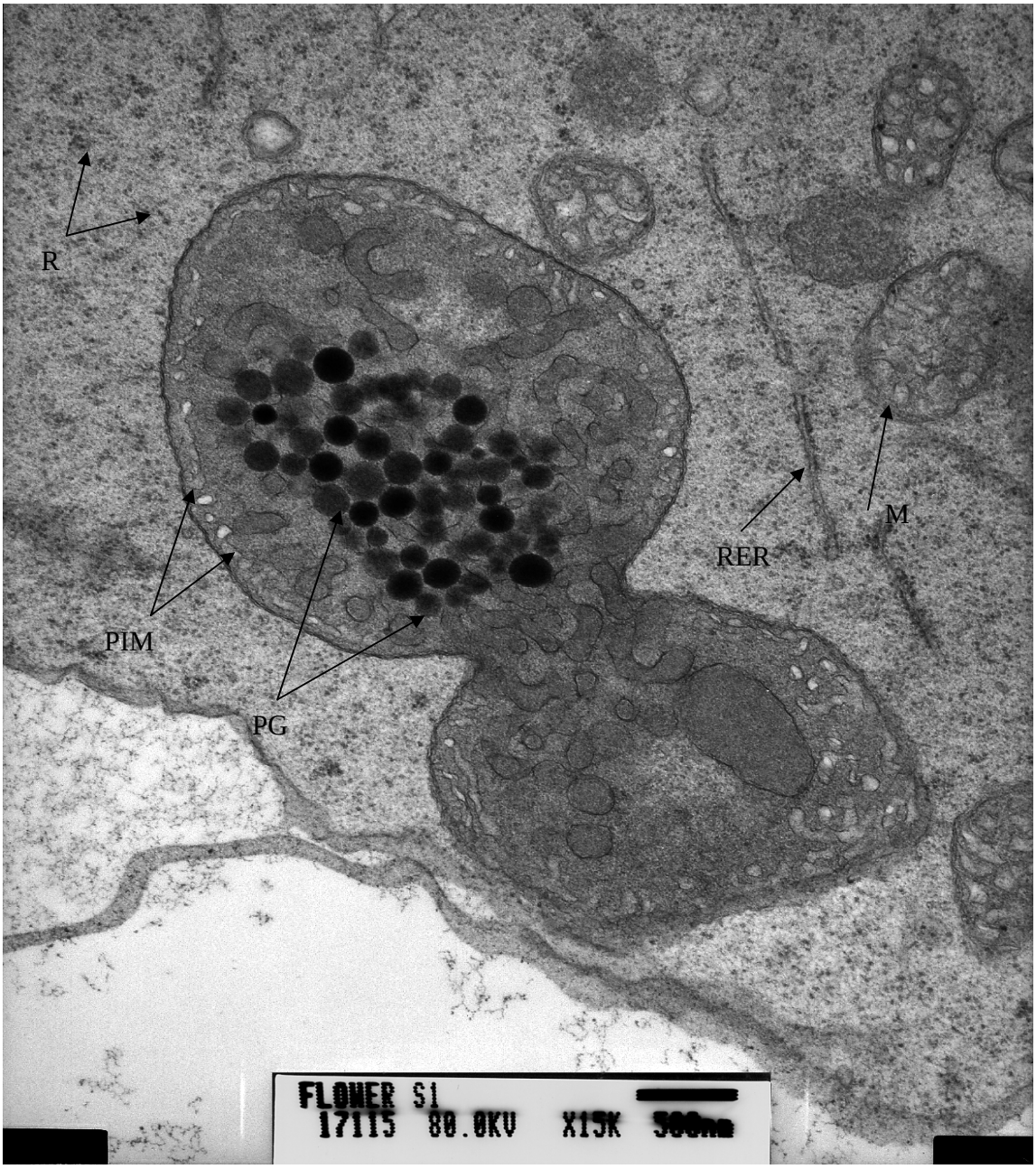


Plate 3. 12 Ultrastructure of an epidermal cell of a gentian petal after sucrose (3%) treatment (magnification = 15000 X). There were plentiful peripheral internal membranes in the plastid shown.

PG, plastoglobuli; PIM, peripheral internal membranes; R, ribosome; RER, rough endoplasmic reticulum; M, mitochondria.

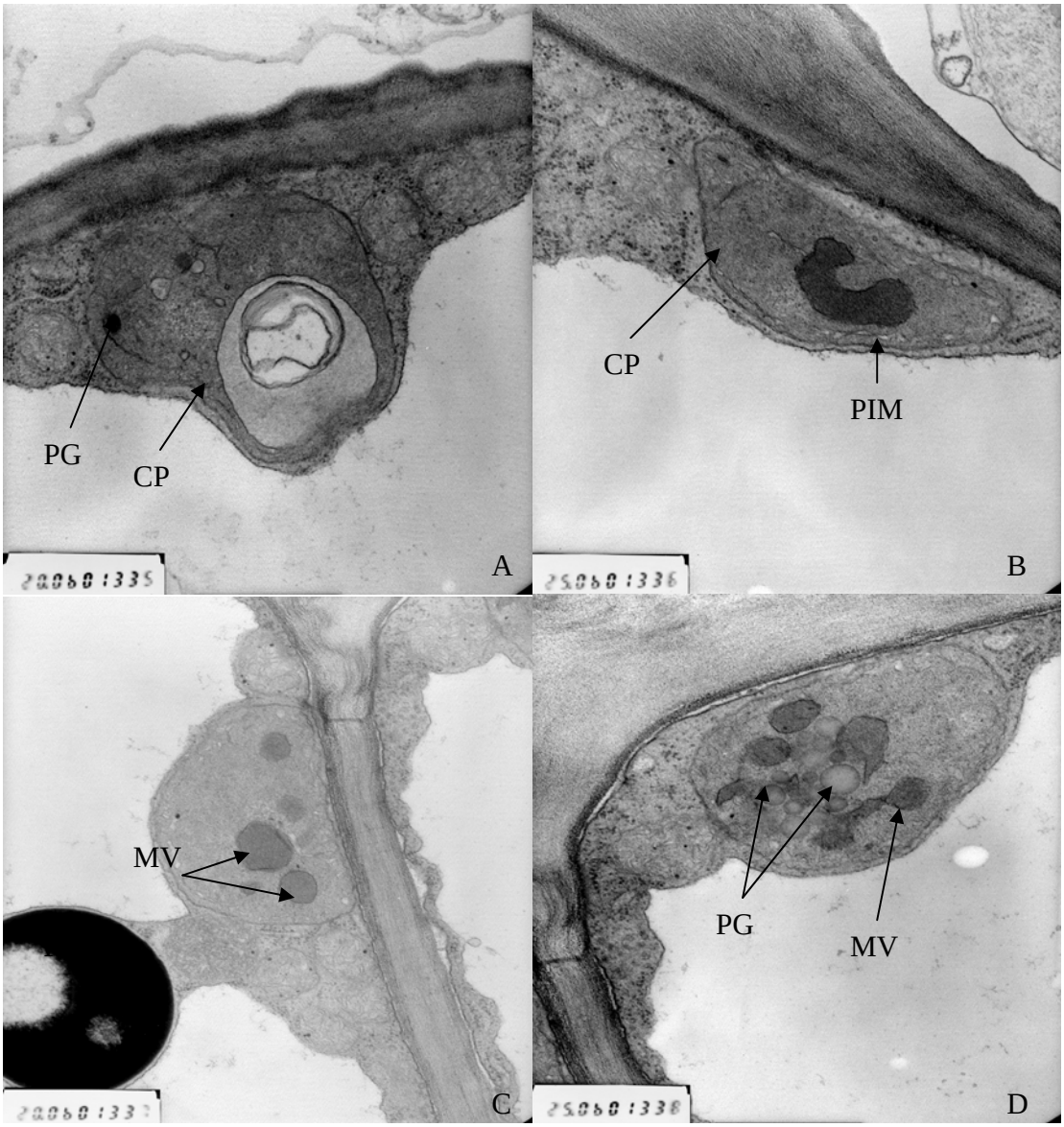


Plate 3. 13 TEM photographs of different chromoplasts formed at the top part of the petal tissue after GA_3 treatment

A and B: special structures appearing in plastids (magnifications = 20000 X and 25000 X, respectively); C: irregular membranous vesicles appearing in plastids (mag. = 20000 X); D: carotenoid-bearing plastoglobuli accumulating around the irregular membranous vesicles (mag. = 25000 X). PIM, peripheral internal membranes; MV, membranous vesicle; CP, chromoplast; PG, plastoglobuli.

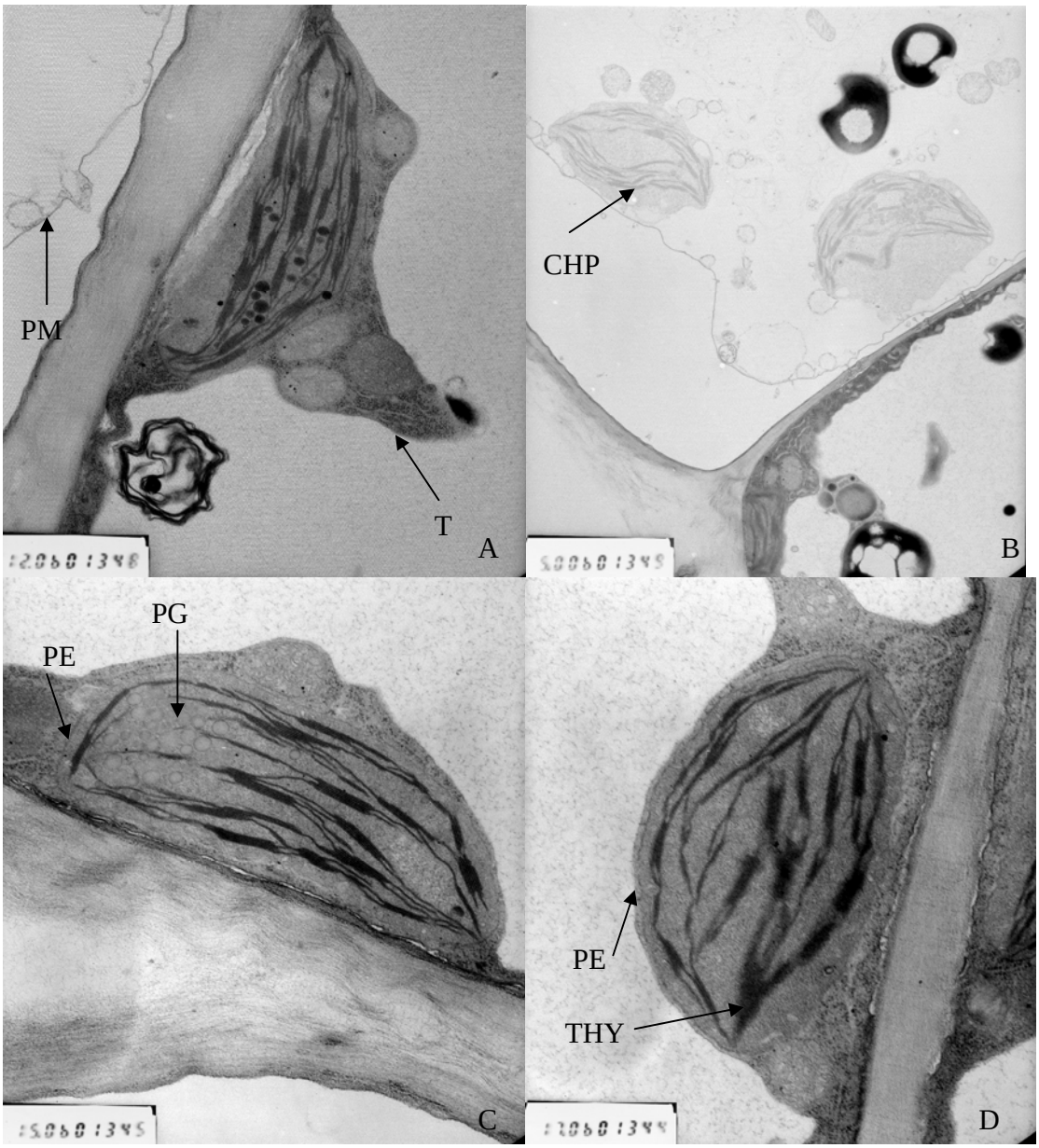


Plate 3. 14 TEM photographs of cells from the other half petal of the same gentian flower used in *Plate 3.13*. The half petal was treated with water.

A, tonoplast rupture (magnification = 12000 X); B, chloroplast degradation (mag. = 50000 X); C, plastoglobuli formation accompanied by thylakoid degradation (mag. = 15000 X); D, thylakoid degradation (mag. = 17000 X). PM, plasmamembrane; T, tonoplast; CHP, chloroplast; PE, plastid envelope; PG, plastoglobuli; THY, thylakoid.

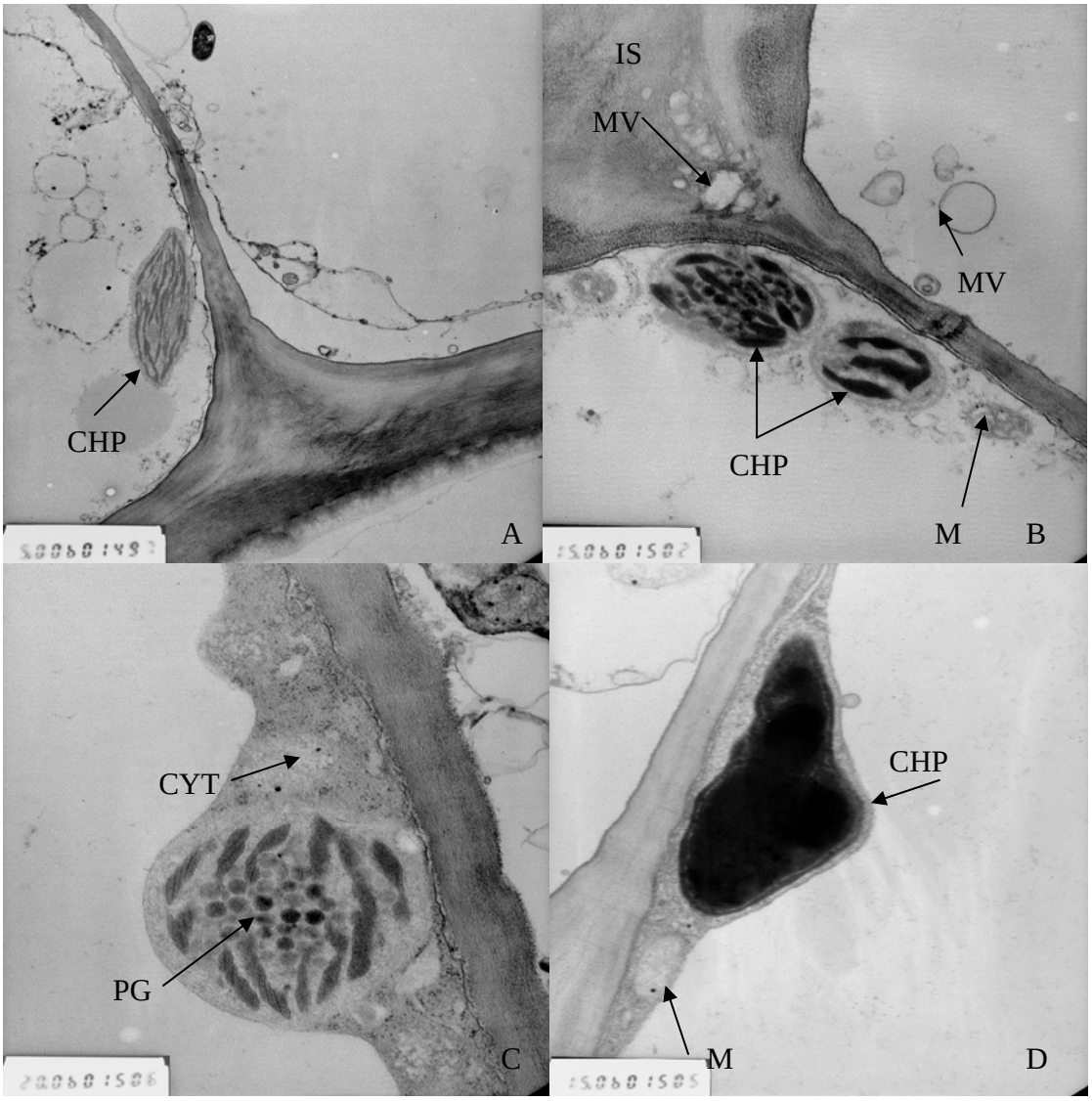


Plate 3. 15 TEM photographs of cells in vein region of the lower part of gentian petals after water or GA_3 treatment. Signs of cytoplasmic degradation were found.

A and B, cytoplasmic degradation in water-treated gentian petals (magnifications = 50000 X and 15000 X, respectively); C, a swollen chloroplast with a lot of plastoglobuli formed in a GA-treated gentian petal (mag. = 20000 X); D, dark dense substance accumulated in a chloroplast of GA_3-treated petal (mag. = 15000 X). M, mitochondria; MV, membranous vesicle; PG, plastoglobuli; CYT, cytoplasm; CHP, chloroplast; IS, internal space.

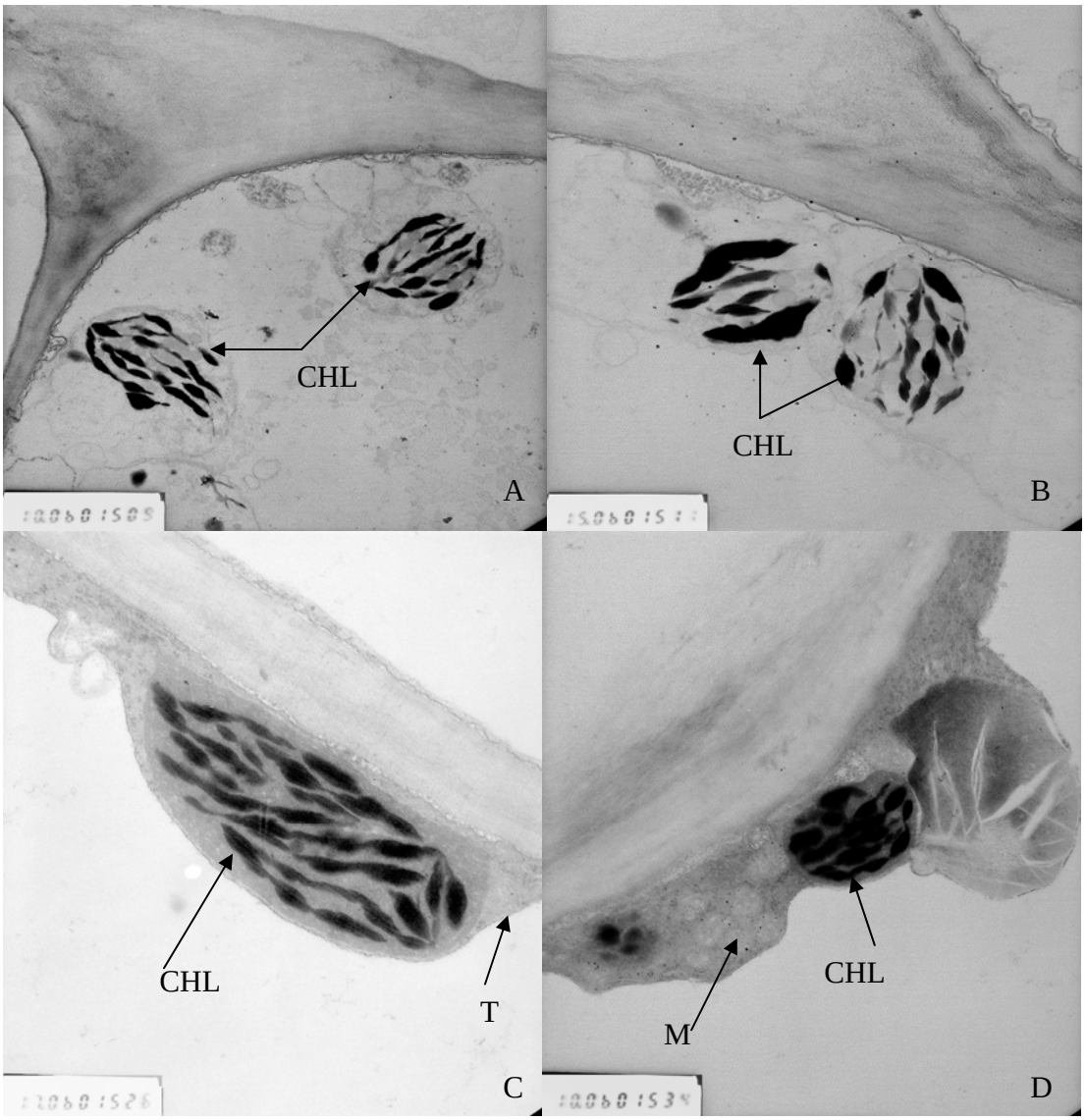


Plate 3. 16 TEM photographs of cells in the vein region of the lower part of gentian petals after water or GA_3 treatment. Closer views of chloroplasts are shown.

A and B, chloroplast degradation in water-treated gentian petals (magnifications = 10000 X and 15000 X, respectively); C and D, chloroplasts were better maintained in GA-treated gentian petals (mag. = 17000 X and 15000 X). CHP, chloroplast; T, tonoplast; M, mitochondria.

3.2.5 Effect of GA_3 treatment on antioxidants in detached gentian flower petals

3.2.5.1 Effect of GA_3 on levels of antioxidant pigments in gentian petals

Interestingly, we found that in the half petal system, not only the vein tissue from the basal part of the GA_3-treated petals was consistently greener, but the bright blue colour of the top part of the petals faded more slowly than that of the control petals as well. In an attempt to estimate the changes of pigment level in response to GA_3 treatment, the pigment content of the petals treated with 5 µM GA_3 was compared with that obtained from control petals. The results showed that in the half petal system GA_3-treated petals had a higher anthocyanin level but there was no significant difference in the contents of chlorophyll a, chlorophyll b, total chlorophyll and carotenoids (*Table 3.7*, upper part). Treatment of detached intact gentian flowers with GA_3 also resulted in slightly higher levels of all the pigments measured (*Table 3.7*, lower part in bold).

Table 3. 7 Effect of GA_3 treatment (5 µM for 24 hours) on chlorophyll contents of isolated half petals (upper part) and detached flowers (lower part in bold)

Pigment content	GA_3 application	Control
CA (µg / g FW)	18.62 ± 4.13 a	13.95 ± 0.88 a
CA (µg / petal)	3.74 ± 0.89 a	2.46 ± 0.07 a
CB (µg / g FW)	30.60 ± 1.10 a	19.85 ± 5.80 a
CB (µg / petal)	6.13 ± 0.24 a	3.64 ± 1.31 a
CT (µg / g FW)	49.22 ± 4.61 a	33.80 ± 5.17 a
CT (µg / petal)	9.86 ± 1.02 a	6.09 ± 1.32 a
CN (µg / g FW)	13.46 ± 2.05 a	11.78 ± 1.66 a
CN (µg / petal)	4.97 ± 0.56 a	3.91 ± 0.20 a
AC (OD 530 nm / petal)	1.30 ± 0.04 a	0.76 ± 0.19 b
CA (µg / g FW)	**14.30 ± 3.19 a**	**11.56 ± 3.35 a**
CA (µg / petal)	**5.25 ± 0.94 a**	**3.69 ± 0.56 a**
CB (µg / g FW)	**22.29 ± 2.75 a**	**17.36 ± 3.12 a**
CB (µg / petal)	**8.27 ± 0.79 a**	**5.72 ± 0.65 a**
CT (µg / g FW)	**36.60 ± 5.33 a**	**21.05 ± 3.47 a**
CT (µg / petal)	**13.52 ± 1.73 a**	**9.42 ± 1.20 a**
CN (µg / g FW)	**13.69 ± 2.64 a**	**11.81 ± 3.65 a**
CN (µg / petal)	**2.83 ± 0.68 a**	**2.21 ± 0.79 a**
AC (OD 530 nm / g FW)	**1.02 ± 0.13 a**	**0.63 ± 0.08 a**

Notes: CA (chlorophyll A); CB (chlorophyll B); CT (total chlorophyll); AC (anthocyanin); CN (carotenoid). Stage 6 flowers (tips of the flowers became wide open) were used. Results (in the same row) assigned with different letters are significantly different ($P<0.05$) using Tukey's method.

3.2.5.2 Effect of GA_3 on antioxidative enzyme activities in gentian petals

The petal of GA_3-treated detached gentian flowers had 44% and 18% more protein based on per petal and per g FW respectively than did untreated petals (*Figure 3.9*). The activities of antioxidative enzymes including SOD, AP and POD were present in gentian petals. On both per FW and flower basis, there were significantly higher activities in SOD and AP, at day 10, for flowers held in GA_3, when compared to water controls (*Figures 3.10 A* and *B*). However, POD activity was similar in both the GA_3-treatment and water control (*Figure 3.10 C*). Statistical analysis also showed that the differences in the activities of SOD and AP on the basis per mg protein between control and GA_3-treated petals were not obvious as well.

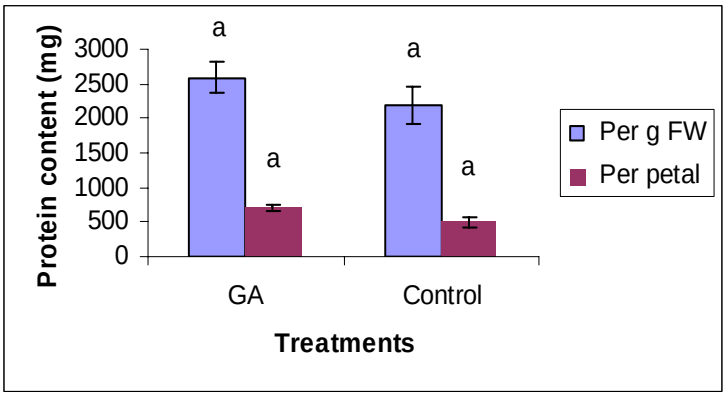


Figure 3. 9 Effect of GA_3 on total protein levels of detached gentian flowers

Gentian flowers (Stage 6) were detached and then held in distilled water or 5 µM GA_3 at room temperature for 24 hours. After this they were held in distilled water in a growth room for two weeks before protein determination. Each treatment consisted of 9 replicate flowers. Vertical bars represent means ± SE.

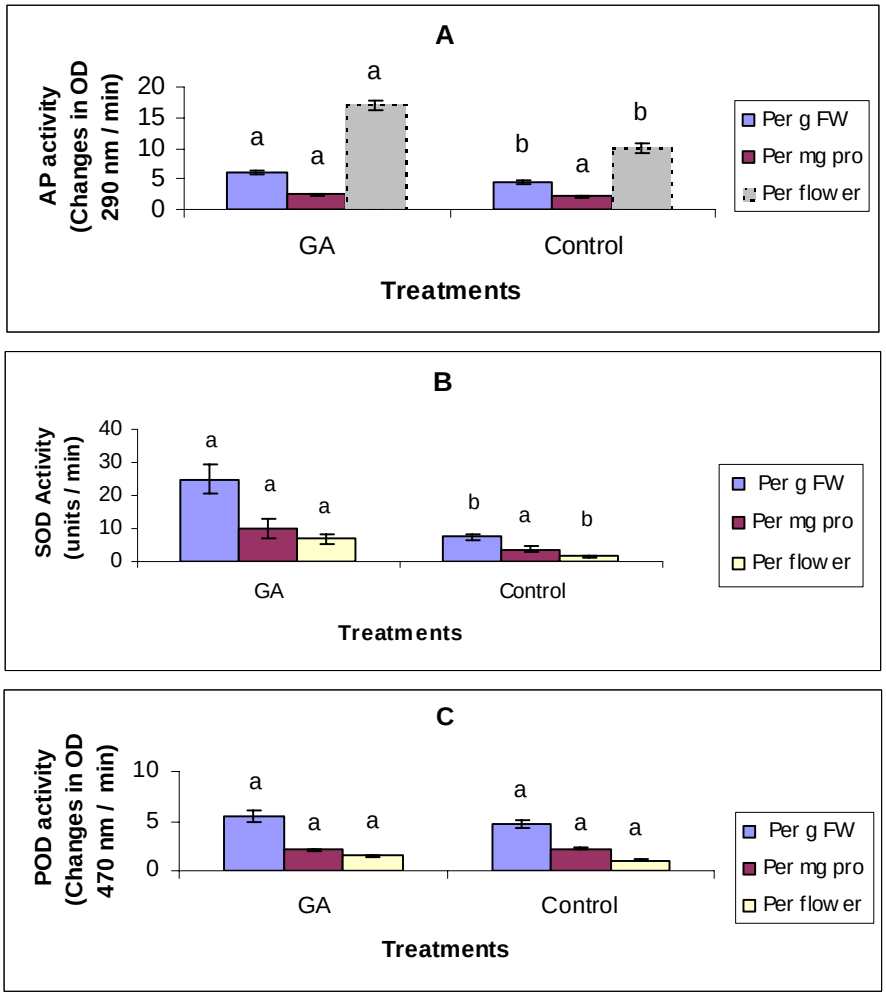


Figure 3. 10 Effect of GA_3 treatment on antioxidative enzyme activities in detached gentian petals

A: AP activity, B: SOD activity C: POD activity of detached gentian flowers. Gentian flowers (Stage 6) were detached and then held in distilled water or 5 µM GA_3 at room temperature for 24 hours. After this they were held in distilled water and kept in a growth room at 23 ºC with continuous lighting for 2 weeks before the appropriate enzyme assays were carried out. (Pro: protein). Each treatment consisted of 9 replicate flowers. Vertical bars represent means ± SE. The bars with different letters indicate that data are significantly different using Turkey's method ($p<0.05$).

3.3 Discussion

3.3.1 Effect of GA_3 on senescence of gentian flowers

For practical purposes, it is necessary to study senescence of flowers borne on a flower stem, which is a very complicated multi-organ system. Buds and flowers at different stages of development simultaneously might exist on the same stem. In addition, the percentage of leaves at various stages of development makes the cut gentian stem a very complicated system for the study of flower senescence. Nonetheless, evidence from the time of the 1st flower to show senescence has been obtained to support the hypothesis that sucrose (3%) and GA_3 (1-10 μM) could delay senescence of the flowers borne on cut gentian stems. Treatment of isolated petals or whole detached gentian flowers with GA_3 also improved their vase life. The beneficial effect of GA_3 on post-harvest longevity had also been demonstrated previously in carnation flowers (Saks and Van, 1993a; Saks and Van, 1993b) and rose petals (Goszczynska et al., 1990). The similar responses among different species suggest that GA_3 might have some general effects on post-harvest life, and a low level of GA_3 might be associated with flower senescence.

The data obtained in this study showed that in the detached single flower system, some of the senescence-associated parameters were significantly suppressed by GA_3 treatment particularly when treated before the flowers were open. In the preliminary experiments, we also observed that when the leaves on the stems seemed to have almost dried out, the GA_3-treated flower stems initiated much more flowers. In the GA_3-treatment, the open flowers appeared much larger in length and width than those of the water control. These observations indicate that the anti-senescence effect of GA_3 was possibly related to its influence on flower initiation and development.

It has been reported that the changes in membrane permeability of petal tissues is related to the appearance of visual signs of cut flower senescence (Kondrat'eva and Belynskaya, 1995). Substantial changes in membrane structure are known to occur well before the

appearance of visual signs of senescence (Paliyath and Thompson, 1990). The data in detached flowers presented here showed that the development of petal electrolyte leakage, a reliable indicator of petal senescence, was significantly retarded by GA_3 treatment. Thus the visual anti-senescence effect of GA_3 in gentian is likely related to its role on the stabilization of the cellular membrane system. In this case, the subsequent production of senescence-associated ethylene might be inhibited since membrane damage is associated with ethylene production. The inhibition of ethylene production by GA_3 has been reported in carnation flowers (Saks and Van, 1993b).

Furthermore, the change in fresh weight is another factor related to the senescence of cut flowers. It was found that the decline in petal fresh weight in gentian was significantly postponed by GA treatment. It is reasonable to assume that the anti-senescence effect of GA is possibly related to the improved water status since water is made up of about 80-90% of the flowers. It has been shown that a significant increase in water content of flowers was observed in cut *Gerbera jamesonii* after GA_3 application (Emongor, 2004).

The process of hydrolysis of carbohydrates has been reported to be associated with petal development and senescence (Zhang and Leung, 2001). A decreased level of starch and increased levels of glucose and fructose were associated with *Alstroemeria* petal senescence (Collier, 1997). Bieleski reported that the onset of fructan hydrolysis concomitant with a large increase in osmoticum is an important event driving flower expansion (Bieleski, 1993). In gentian, GA_3 could increase the osmolality of gentian petal cell sap through promoting hydrolysis of carbohydrates such as starch and sucrose into fructose and glucose. The accumulation of these solutes would improve the ability of petals to uptake water from the holding solution and provide a better water balance for petal life.

Moreover, respiration is one of the various important events affecting flower vase life (Bhattacharjee and Pal, 1999). The regulation of GA on respiration and the associated events might contribute to the delayed senescence of GA_3-treated gentian petals, compared to the water control. A lower respiration rate in petals may be involved since

decreased respiration in lily leaf and *Arabidopsis* mitochondria were observed following GA_3 application (Franco and Han, 1997; Millar et al., 2003). Furthermore, the induction of the hydrolysis of complex carbohydrates to reducing sugars in plants by GA_3 treatment might provide more respiratory substrates for petal life.

It has been previously reported that GA_3 application significantly delays leaf yellowing and increases the chlorophyll level in leaves of cut flowers when compared to the control (Hicklenton, 1991; Jordi et al., 1993). This suggests that a positive role of GA_3 on the level of chlorophyll in the leaves of flower stems may also be related to the anti-senescence effect of GA_3 on gentian flowers on cut stems.

3.3.2 Colour changes in response to GA_3 treatment

Anthocyanins are generally located in the vacuole of epidermal cells of a plant (van-Doom, 2004). They are the largest group of plant pigments responsible for colours ranging from red to violet and blue (Vaknin et al., 2005). Data from the present study showed that a decrease in the level of anthocyanins was associated with gentian petal senescence. GA_3 application significantly retarded petal discoloration and senescence, indicating a possible positive role of GA_3 treatment on anthocyanin level. Pigment analyses supported this, although a statistically significant difference was only found in the half petal system.

Enhanced synthesis of anthocyanin by GA_3 treatment in gentian petals is possible since GA_3 was reported to increase the expression of structural genes in anthocyanin biosynthesis (Wang et al., 2003). In addition, the factors involved in the process of anthocyanin degradation should also be considered. For example, reducing agents such as DTT and glutathione were reported to inhibit anthocyanin degradation (Vaknin et al., 2005). The higher activities of SOD and AP antioxidant enzymes in GA_3-treated petals may provide a more reduced cellular environment and influence the degree of oxidation of anthocyanin. It has been shown that a peroxidase was likely to be involved in the

degradation of anthocyanin in *Brunfelsia calycina* flowers (Vaknin et al., 2005). The results of POD activity in this study do not support this, however.

Cell sap pH is another factor that could affect the striking visible changes of gentian petal colour from fresh bright blue (due to the presence of anthocyanin) to browning prior to petal wilting. Previous studies in rose petals have shown that an increase in cell sap pH was associated with the immediate effect of sucrose in delaying visual colour change (Oren et al., 2001). Anthocyanin degradation was correlated with changes in the vacuolar microenvironment, such as pH, concentration of co-pigments or metals (Vaknin et al., 2005). Data from this study showed that in gentian the change of petal color from blue to brown was associated with a decreased level of cell sap pH occurring in gentian petals during senescence (*Table 3.2*). This change in pH is assumed to be related to the organic acids such as aspartic, malic, and tartaric acids that are released from protein degradation (Thimann, 1980). It would seem worthwhile in future studies to confirm if the anti-senescence effect of GA_3 in gentian petals is associated with cell sap pH level of petals.

3.3.3 Ultrastructural changes in response to GA_3 treatment

Most of the general senescence-associated features including an increase in the number and size of plastoglobuli, swelling of thylakoids, large lipid deposits and cellular degradation were observed in the gentian petals used in the present study. These results were very similar to those obtained by other researchers (Smith et al., 1992). In this study, we mainly examined the changes of plastids including chloroplasts and chromosplasts. The latter are morphologically characterized by the absence of thylakoids and are specialized to sequester and accumulate carotenoids (Giuliano et al., 1993).

Generally the ultrastructure of chromoplasts has been classified into several categories: globular, fibrillar, tubular, crystalline and membranous. Observations in this study indicate that the disorganized, appressed membranous vesicles in plastids could be lipophilic precursors to plastoglobuli.

Since the breakdown of the thylakoid system and the appearance of plastoglobuli occurred at the same time in a chloroplast, it seems likely that the two processes were in some way connected. The material from the disappearing thylakoids might be used directly in the production of plastoglobuli. In this way, the transformation of chromoplasts from chloroplasts is also possible in gentian petal cells since complete degradation of thylakoids at the final stage may result in the formation of globular chromoplasts.

One of the major changes in the ultrastructure of gentian petals was that chromoplast biogenesis was promoted by GA_3 application. It has been reported that chlorophyll and carotenoids are synthesized in a quantitatively and qualitatively coordinated manner in chloroplasts. When this balance between chlorophyll and carotenoids is strongly changed in favor of carotenoids, the plastid ultrastructure may change (Rabbani et al., 1998). In this case, a relatively higher ratio of carotenoid level / chlorophyll level possibly indicates chromoplast formation. This may lead to inhibition of the normal assembly of thylakoid membranes and may shift the development of plastids toward the formation of chromoplasts.

The enhancement of GA_3 on chromoplast formation possibly indicates an influence of GA_3 on the ratio of carotenoids to chlorophyll. Results from pigment analyses showed that the senescence associated decrease in photosythetic pigment (chlorophylls and carotenoids) content in gentian petals was slowed down by GA_3 but not arrested, which is consistent with the results in cut carnation (Saks et al., 1993b). The anticipated significant increase in level of carotenoids was not observed. Probably a bigger sample size and more different sampling times in further investigations would be required to clarify this.

It has been shown that free carotenoids are predominantly located in the plastoglobuli during senescence (Biswal, 1995). It has also been reported that ‘chromoplast internal structure’ (CIMs) contain carotenoids (Bonora et al., 2000). Moreover, GA_3 could up-regulate the expression of chromoplast-specific carotenoid-associated proteins (CHRC), which are associated with the lipoprotein complexes in chromoplasts (Vainstein et al.,

1994; Vishnevetsky et al., 1997). Taking together, these observations suggest that enhanced levels of antioxidant carotenoids might be related to the anti-senescence effect of GA_3 on gentian petals.

3.3.4 Antioxidative enzymes changes in response to GA_3 treatment

SOD and AP are known to be important ROS-scavenging enzymes involved in the detoxification of superoxide and hydrogen peroxide in plants. It was of interest to determine whether the anti-senescence effect of GA_3 is linked with a change in the activities of these antioxidative enzymes. The present results showed that significantly higher activities of AP and SOD were found in GA_3-treated gentian petals than the water control (*Figures 3.15 A* and *B*).

It has been shown that a decline of ascorbate peroxidase activity is a perrequisite factor for tepal senescence in gladiolus (Hossain et al., 2006). The higher level of AP activity in GA_3-treated gentian petals suggests that there was an enhanced capacity of petals to eliminate the H_2O_2 generated during metabolism. In addition, ascorbate acts as a general ROS scavenger in plants and directly reacts rapidly with $O_2^{\cdot -}$, $OH^{\cdot}$ and singlet oxygen. More ASC might be produced in GA_3-treated gentian petals since it has been reported that more ASC is produced in mitochondria in response to GA_3 treatment (Millar et al., 2003).

With regard to SOD, a few studies have provided similar evidence of the anti-senescence effect of GA_3 on SOD in leaves and fruits. It has been reported that delaying senescence of *Rumex* leaf discs by GA_3 was related to the retardation of a decline in SOD activities and an increase in the level of lipid peroxidation (Dhindsa et al., 1982). GA_3 can also regulate the expression of SOD genes. In tobacco, an approximately two-fold increase in the levels of the chloroplast-specific SOD transcripts was induced following GA_3 treatment (Kurepa et al., 1997).

Several studies have found that the activities of proteases and ubiquitin (to target the bound protein for degradation by a specific protease) increase during flower senescence (Sabehat and Zieslin, 1994; Stephenson and Rubinstein, 1998). The senescence-related protease activities has also been reported to be inhibited in several plants following GA_3 application (Cercos et al., 1999; Eason, 2002a). It is an interesting topic for further investigations to examine the effect of GA_3 on proteolytic degradation of the antioxidant enzymes in gentian petals.

It is unclear if co-ordination of antioxidant responses to various kinds of stresses, including senescence-related oxidative stress, involves gene regulation. Changes in antioxidant enzyme activities do not necessarily correlate with corresponding changes in the abundance of particular transcripts. Despite an increase in the activities of SOD and AP, we do not know whether there are increases in the level of the transcripts encoding the enzymes in response to GA_3 treatment. This requires further investigations.

Little information is available on the mechanisms of the anti-senescence effect of GA_3 on flowers. The results obtained in the present study support the contention that GA_3 could improve the post-harvest quality of gentian flowers, at least in part by affecting antioxidant enzyme activities. GA_3 significantly influences the activities of SOD and AP while the effect on antioxidant pigments is relatively minor. An effective antioxidant defence enhanced by added GA_3 could counteract oxidative stress and therefore reduce oxidative damage in cut gentian flowers.

CHAPTER 4

RELIEF OF OXIDATIVE STRESS IN PETUNIA PETALS BY ETHANOL TREATMENT

4.1 Introduction

In plants, high levels of reactive oxygen species (ROS) are harmful to living organisms since they could oxidise biomolecules such as DNA, proteins and carbohydrates and lipids. Various antioxidants are required to protect living cells against oxidative stress / oxidative damage (Tanyolac et al., 2007). Recent studies, though only on a couple of flowers, have led to a suggestion that elevated oxidative stress and probably down-regulation of antioxidative defense reactions are associated with ethylene production and senescence (Panavas and Rubinstein, 1998a; Zimmermann and Zentgraf, 2004). If oxidative stress is a key regulatory event leading to flower senescence, it is reasonable to expect that diverse post-harvest treatments may influence oxidative metabolism in cut flowers.

Few reports are available regarding the effect of post-harvest treatments on the oxidative / antioxidative metabolism of cut flowers. With regard to the anti-senescence effect of ethanol, a free radical scavenger (Dhindsa et al., 1982), most of the previous reports investigated its linkage with the pathway of ethylene (Lee and Kim, 1999; Podd et al., 2002a; Pun and Rowarth, 2001; Pun et al., 1999). The objectives of this study were firstly to confirm the anti-senescence effect of ethanol on petunia petals. Secondly, a special

emphasis is placed on whether ethanol may enhance the tolerance of petunia petals towards senescence-associated oxidative stress. This may be due to an alteration of the capacity of the tissue to scavenge reactive oxygen species. Therefore, the changes of antioxidative defense enzymes (SOD, AP and POD) and several compounds that may function as antioxidants were studied. For example, carotenoids can protect green tissues against photo-oxidation. They can actively quench singlet oxygen and minimize singlet oxygen formation by absorbing excess energy from excited triplet states of chlorophylls (Tapiero, 2004). Another well-known antioxidant, ascorbic acid, can react with superoxide and hydroxyl radicals and scavenge singlet oxygen (Bashor and Dalton, 1999). The effect of ethanol on chlorophyll contents was analysed as well since they are involved in scavenging or generation of oxygen stress (Helsper et al., 2003).

4.2 Results

4.2.1 Development and senescence of petunia flowers under glasshouse conditions

4.2.1.1 Changes in fresh weight and electrolyte leakage

Development and senescence of petunia flowers were often studied at three major stages, namely buds, open flowers and senesced or wilted flowers. However, more detailed descriptions about flower development and senescence are shown in *Plate 2.2*. As shown in *Figures 4.1 A* and *B*, the fresh and dry weights of petunia flowers increased continuously during bud growth (from Stages 1 to 4) and flower development (Stages 5 to 7). After reaching a maximum at Stage 8, they started to decrease as flowers senesced (Stages 9 to 12). Petal senescence was manifested by a significant increase in petal electrolyte leakage at Stage 10. Prior to this, there was a constant and lower level of electrolyte leakage from the petal (*Figure 4.1 C*).

Ovary weight varied little during the first four stages but there was a significant increase from Stage 4 to Stage 5 (*Figure 4.1 D*). During the later petal senescence periods, the fresh weight of ovaries did not change very much. This was different from the trend of a continuous increase observed during petal senescence in gentian (*Table 3.2*).

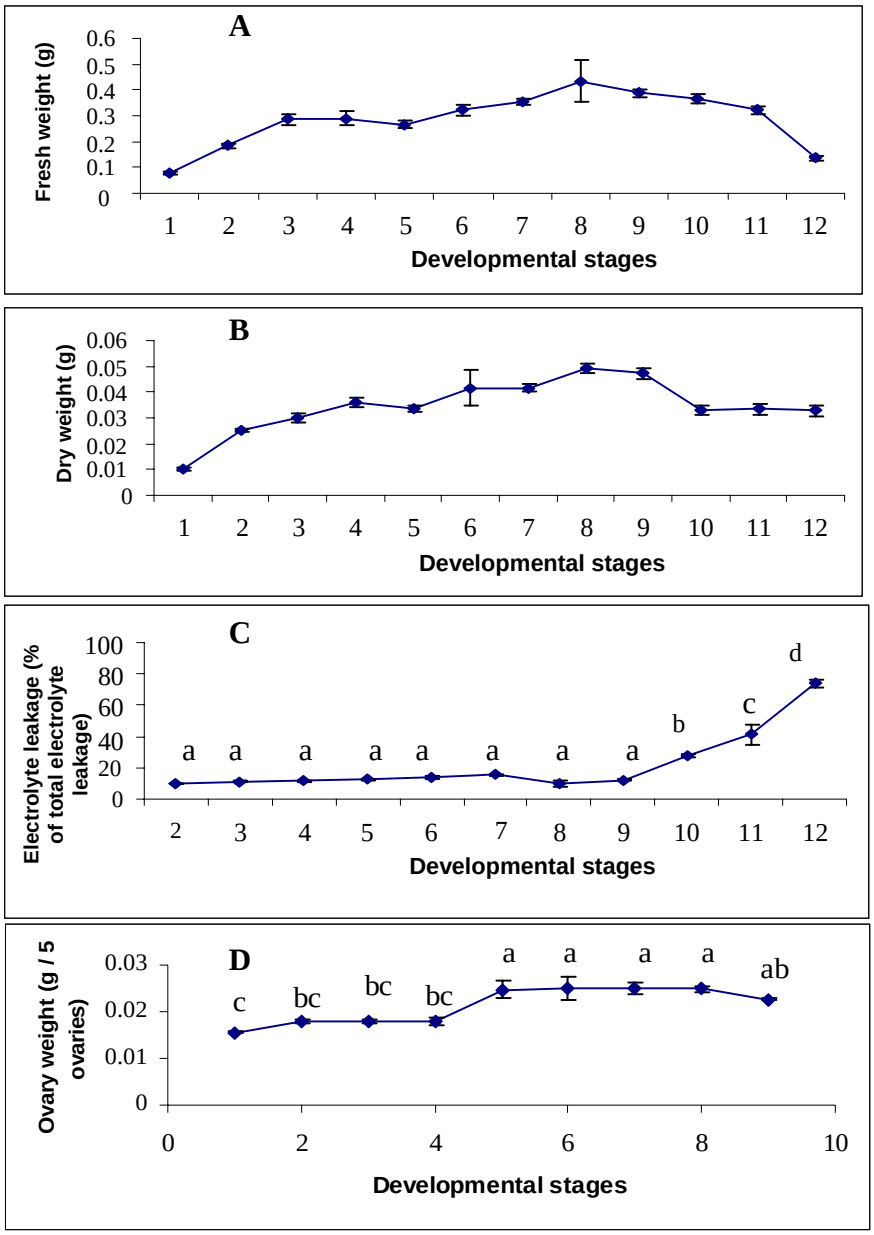


Figure 4. 1 Changes associated with development and senescence of petunia flowers under glasshouse conditions

A: fresh weight (g / petal); B: dry weight (g / petal); C: petal electrolyte leakage (%); D: ovary weight (g / 5 ovaries). Means ± SE (vertical bars) from 3, 5 and 10 individual flowers for petal electrolyte leakage, ovary weight and petal weight measurements, respectively. Different letters (a, b, c and d) indicated that the results are significantly different ($p<0.05$) using Tukey's method following ANOVA.

4.2.1.2 Changes of isozyme profiles during petal development and senescence

Five developmental stages including bud, half-open flower, open flower, 'beyond open flower' and old flower were selected for this study. Petals at the appropriate stages were harvested from petunia plants grown in the glasshouse. The changes of selected antioxidative enzymes during petunia flower development and senescence were studied using non-denaturing PAGE.

4.2.1.2.1 Superoxide dismutase (SOD)

SOD constitutes the first line of defense against oxidative stress by removing superoxide radicals before they can react with hydrogen peroxide and give rise to the extremely reactive hydroxyl radical. It plays a very important role during petunia petal development and senescence. Following electrophoretic separation of extracts from petals of different developmental stages, 4 major bands (designated S_1, S_2, S_3, and S_4) were observed and indicated on *Plate 4.1 A*. The types of SOD could be distinguished by pre-treating the native gels after electrophoresis of crude petunia petal extracts in 3 mM KCN or 5 mM hydrogen peroxide for 20 minutes in the dark before staining for SOD activity (Droillard and Paulin, 1990). S_1 seems to be a Mn-SOD as it remained after hydrogen peroxide pre-treatment (*Plate 4.2*). S_3 might be a Cu-Zn-SOD because it was inhibited by both hydrogen peroxide and cyanide. Both S_2 and S_4 could be Fe-SODs, which were inhibited by hydrogen peroxide but not cyanide.

Overall, among the SOD isoforms, the activity of iron-SOD (S_2) was the highest at different developmental stages. It was the main component of petunia petal SOD isoenzymes and its activity was altered slightly throughout petal development and senescence (*Plate 4.1 A*). In contrast, the intensities of other isoforms S_1, S_3 and S_4 decreased from the onset of petal senescence (Stage PS) when compared to their intensities at Stage FO. However, the difference in SOD isozyme intensities at different

senescence stages was slight (*Plate 4.1 A*). These trends were clearly confirmed in the replicate experiment with same tissue load per lane (*Plate 4.1 B*).

4.2.1.2.2 Ascorbate peroxidase (AP)

Five electrophoretic forms of AP activity were found regardless of whether the crude petal extracts were prepared in the presence or absence of ascorbate (*Plates 4.3 A* and *B*). These isoforms are numbered A_1, A_2, A_3, A_4 and A_5 based on their anodal migrations. A_1 was a very thin and clear band on top of the gels. A_2, A_3 and A_4 were the common main bands in petunia petals. A_5 was more diffuse and obscure to be observed, probably due to quicker degradation. A_1 and A_4 isozymes were absent or present with very low activities at the bud stage and then increased a little in half-open flowers (*Plate 4.3*). Most of the AP isozyme activities greatly increased as a petal fully opened, and this increase continued until the maximum activity period (Stage OF or PS). Then the activities of most of them, particularly the main band A_3, decreased during the onset of the petal senescence (Stage HS) as petals had lost water and become soft. There was no obvious difference in the pattern between the cytosolic and other forms of AP isozymes.

4.2.1.2.3 Peroxidase (POD)

When crude enzyme extracts of petunia petals were analysed on 12.5% anodic native PAGE gels, seven isoforms (designated $P_{1\text{-}7}$) were detected (*Plate 4.4*). P_1 and P_2 were either absent or scarcely detectable before petal senescence. P_3 was the common and main band of POD isozyme observed during petunia petal development and senescence. The activities of $P_{4\text{-}7}$ were high at Stages OF and PS. P_7 appeared when flowers were half open.

From Stage PS to Stage HS, there appeared to be a general decline in POD activity (*Plate 4.4 A*). However, the increases in the intensities of all the bands of POD isozymes were observed from Stage HS to Stage SF.

Another experiment for equal petal tissue load in each lane showed that there was a continuous decrease of POD isozyme activity possibly with the exception of P_3 at Stage SF (*Plate 4.4 B*). Therefore, the superficial increase of POD activity at this last senescence stage observed in *Plate 4.4 A* was probably due to decreased fresh weight of the petal.

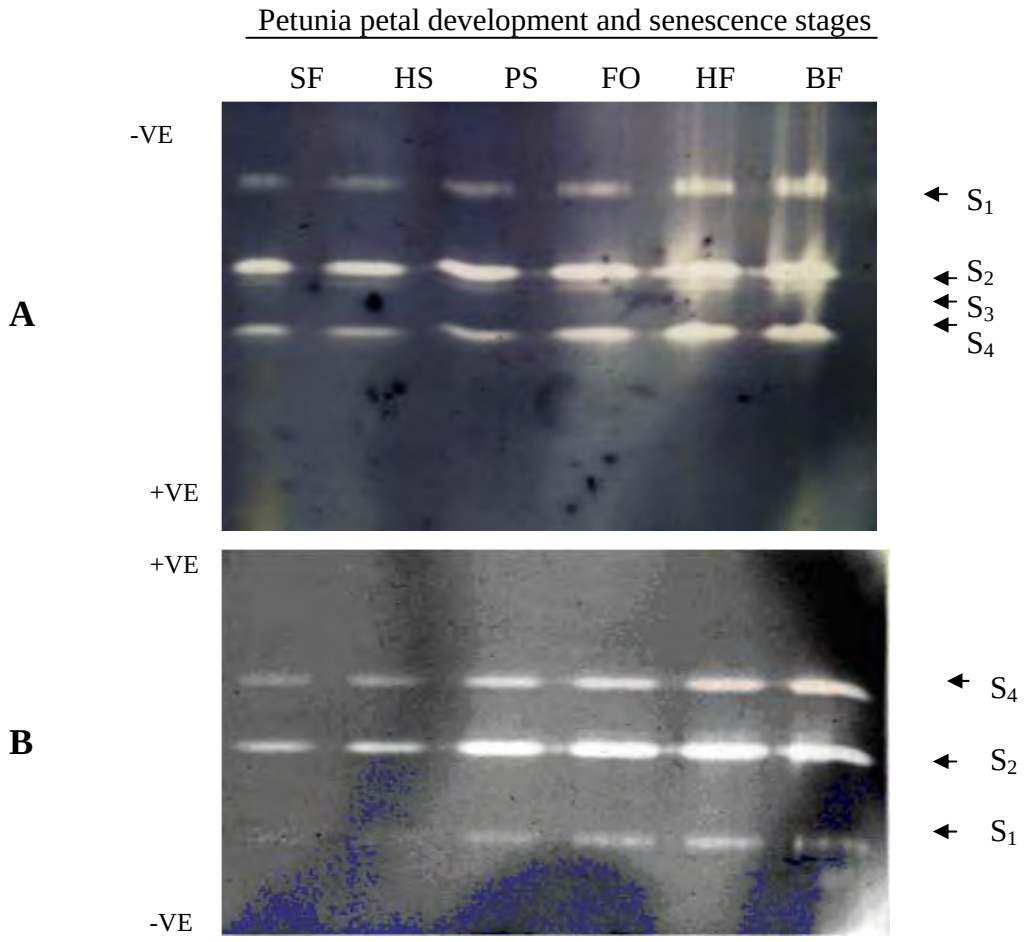


Plate 4. 1 Native PAGE analysis of SOD isoforms in crude petunia petal extracts during petal development and senescence

Different lanes represent petal extracts from six developmental and senescence stages: bud flower (BF); half-open flower (HF); open flower (OF); pre-senescence (PS): part of the petal starting to drop; half senesced flower (HS): part of the petal edge curled up; senesced flower (SF): part of the petal tissue appeared to be crispy. For A and B in each lane with samples from BF, HF and OF, crude extracts from the same fresh weight of petal were loaded. For A, in each lane with samples from PS, HS and SF, crude extracts from the same fresh weight of petal were loaded. However, for B, in each lane with samples from PS, HS and SF, crude extracts of the same tissue load were applied.

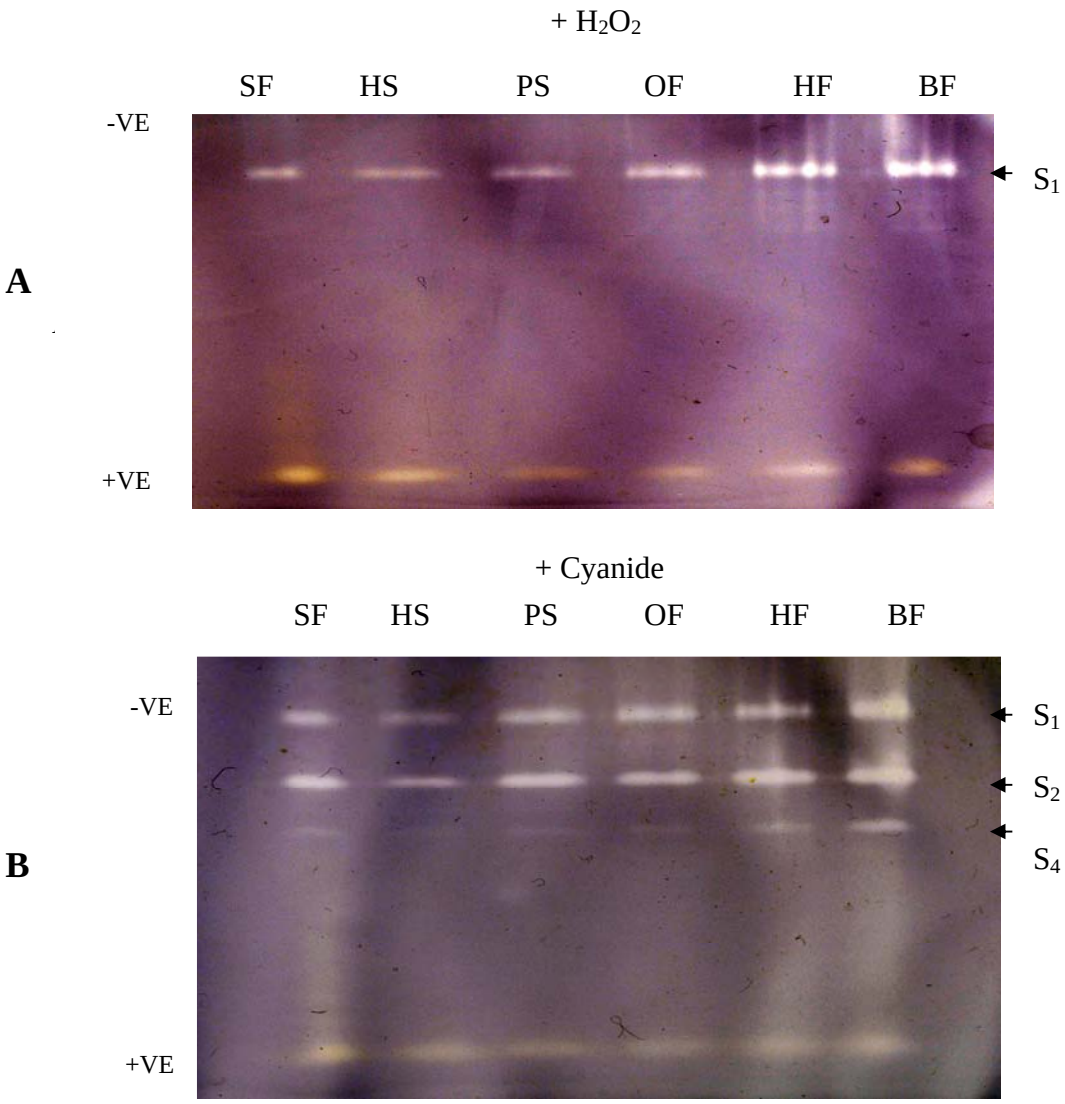


Plate 4. 2 Native PAGE gels for identifying isoforms of SOD

After native PAGE of crude extracts of petunia petal, the gels were pre-treated in A: 5 mM H_2O_2 and B: 2 mM KCN and then followed by staining for SOD activity for 20 minutes in the dark. Lanes 1-6: petal extracts from 6 developmental and senescence stages: senesced flower (SF): part of the petal tissue appeared to be crispy; half senesced flower (HS): part of the petal edge curled up; pre-senescence (PS): part of the petal starting to drop; open flower (OF); half open flower (HF) and bud flower (BF). The bands at the bottom of the gels were low-molecular-weight interference substances and not SOD isozymes.

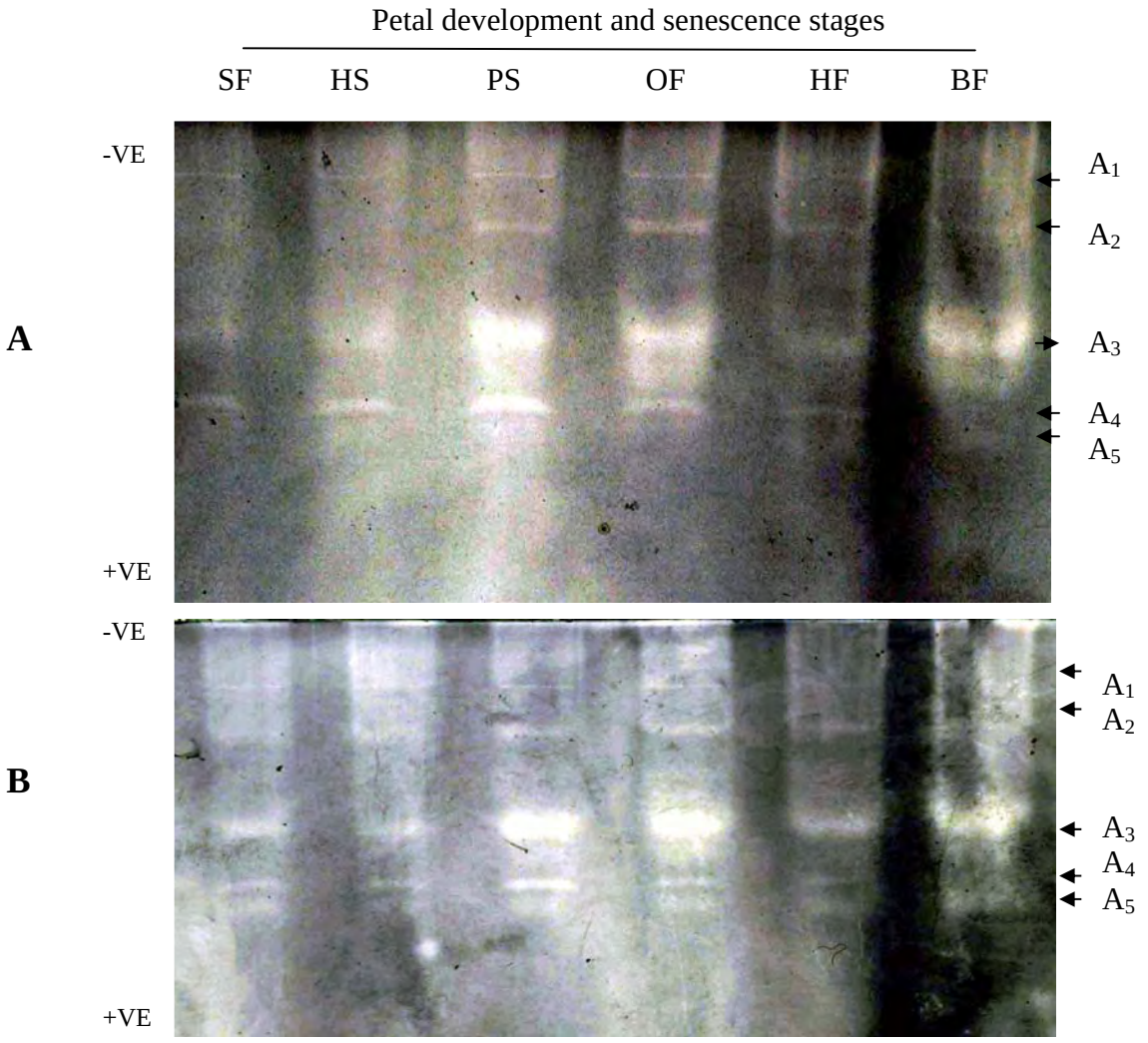


Plate 4. 3 Native PAGE analysis of ascorbate peroxidase isoforms in crude petunia petal extracts during petal development and senescence

Flower development and senescence stages: senesced flower (SF): part of the petal tissue appeared to be crispy; half senesced flower (HS): part of the petal edge curled up; pre-senescence (PS): part of the petal starting to drop; open flower (OF); half-open flower (HF) and bud flower (BF). A: 20 minute staining for AP activity when enzymes were extracted in the presence of ascorbate; B: 30-minute staining for AP activity when enzymes were extracted in the absence of ascorbate.

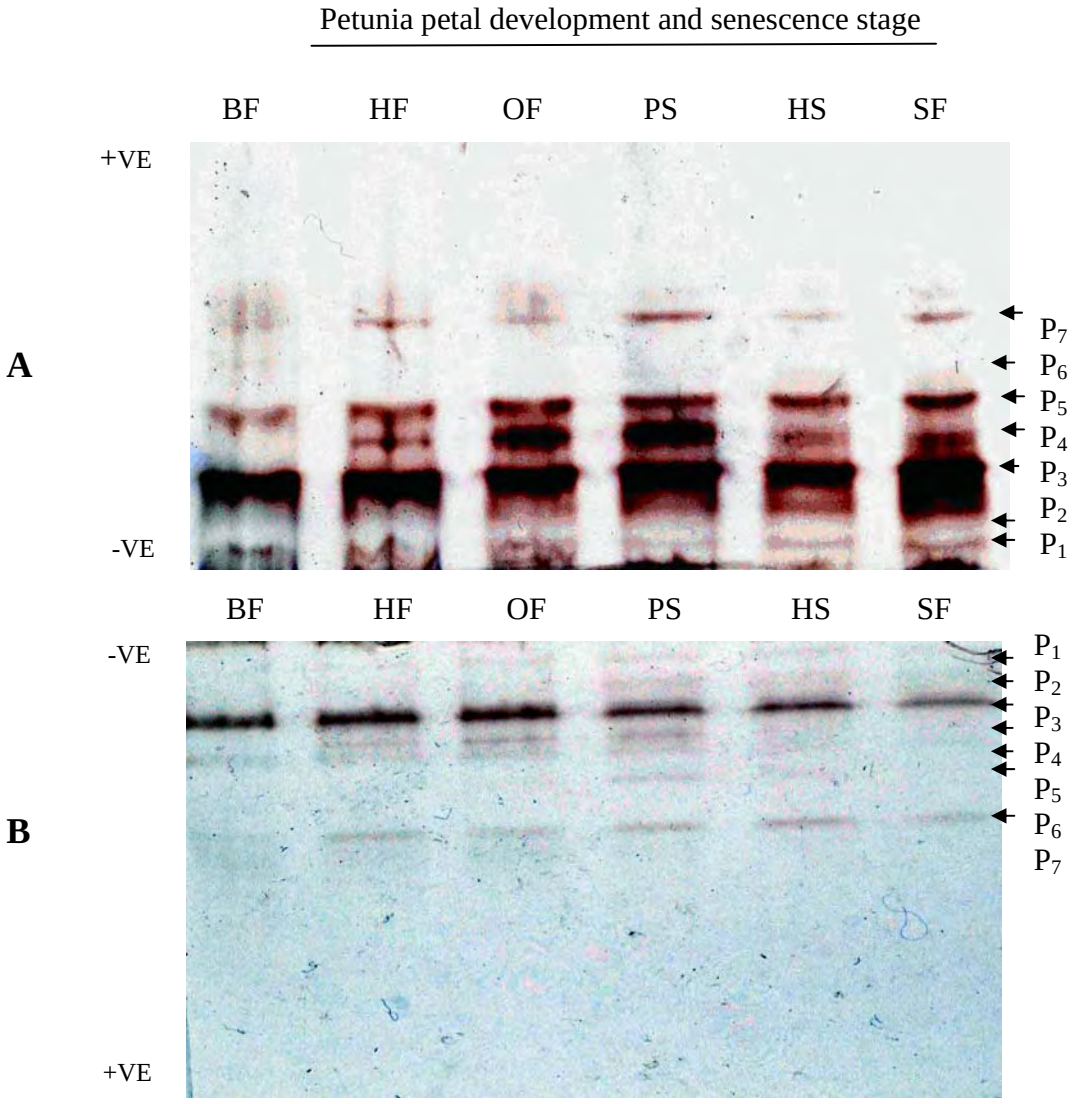


Plate 4. 4 Native PAGE analysis of peroxidase isoforms in crude petunia petal extracts during petal development and senescence

Different lanes represent petal extracts from six development and senescence stages: BF, bud flower; HF, half-open flower; OF, opened flower; PS, pre-senescence (part of the petal starting to drop); HS, half senesced flower (part of the petal edge curled up); SF, senesced flower (part of the petal tissue appeared to be crispy). Samples from BF, HF and OF were same FW load while samples from 3 senescence stages, PS, HS and SF, were same fresh weight load in A but same tissue load in B.

4.2.1.3 ROS changes associated with development and senescence of petunia petals under glasshouse conditions

Petal senescence was hypothesized to be accompanied by oxidative stress, which is generally known to be associated with an increased ROS level. Petunia petals were imbibed in DAS for 8 hours or NBT for 8-13 hours, DAS polymerized to form a deep brown product in the presence of H_2O_2, and the reaction of NBT with superoxide resulted in the deposition of purple formazan. The regions of hydrogen peroxide and superoxide formation in different developmental and senescence stages of petunia petals were identified and assessed qualitatively based on the intensity of the coloration from the photographed images.

The present results showed that accumulation of superoixide was detected at the early senescence stage (PS) (*Plate 4.5*). Stage HS petals showed clear formazan formation because of more superoxide accumulation (*Plate 4.5 B, middle*). With the method used in this study, H_2O_2 was most notable at the later senescence stage HS_2 (*Plate 4.6*). For both superoxide and hydrogen peroxide staining, there seems to be less in SF than HS.

4.2.1.4 Changes of electrolyte leakage from different parts of petunia petals

Selected Stage PS or HS petals were separated transversely into three portions (top, middle and bottom) in order to show the differences in the process of senescence among different parts of a petunia petal. There was no difference in level of electrolyte leakage among different parts of a petal at Stage PS when the petal was still healthy (*Figure 4.2*). However, at Stage HS when the petal tissue started to lose water, the rate of electrolyte leakage in all three parts of the petals increased. Senescence of the top part of the petal was earlier since electrolyte leakage from this part was significantly higher than the other two parts.

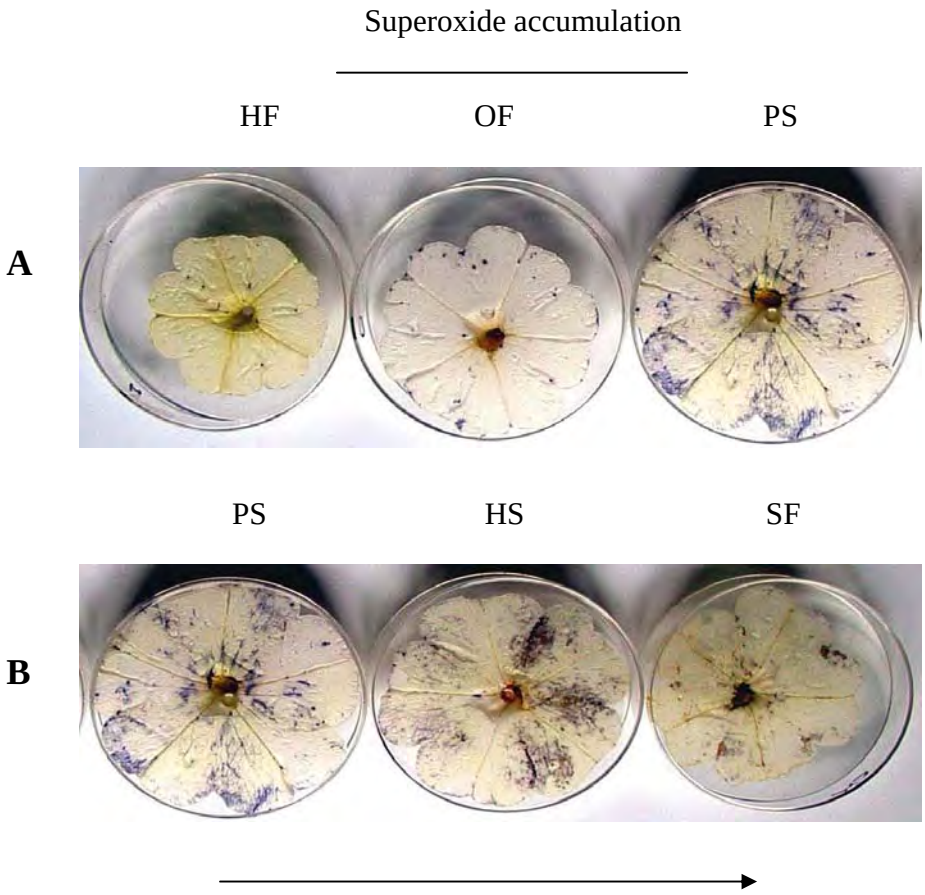


Plate 4. 5 Localization of superoxide accumulated in petunia petals at different stages of development (A) and senescence (B)

Notes: half open flower (HF); opened flower (OF); pre-senescence (PS): part of the petal starting to drop; half senesced flower (HS): part of the petal edge curled up and senesced flower (SF): part of the petal tissue appeared to be crispy.

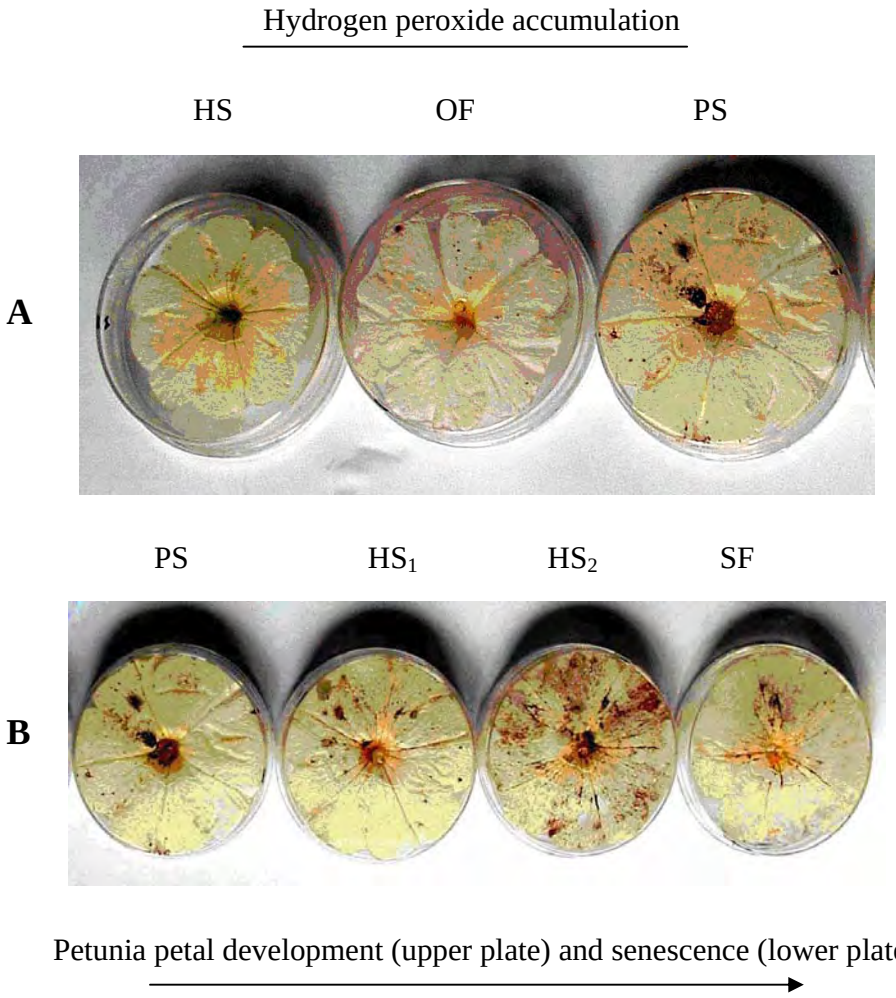


Plate 4. 6 Localization of hydrogen peroxide accumulated in petunia petals at different stages of development (A) and senescence (B)

Notes: half open flower (HF); opened flower (OF); pre-senescence (PS): part of the petal starting to drop; half senesced flower (HS): part of the petal edge curled up and senesced flower (SF): part of the petal tissue appeared to be crispy. Stage HS_2 is a developmental stage relatively later than Stage HS_1.

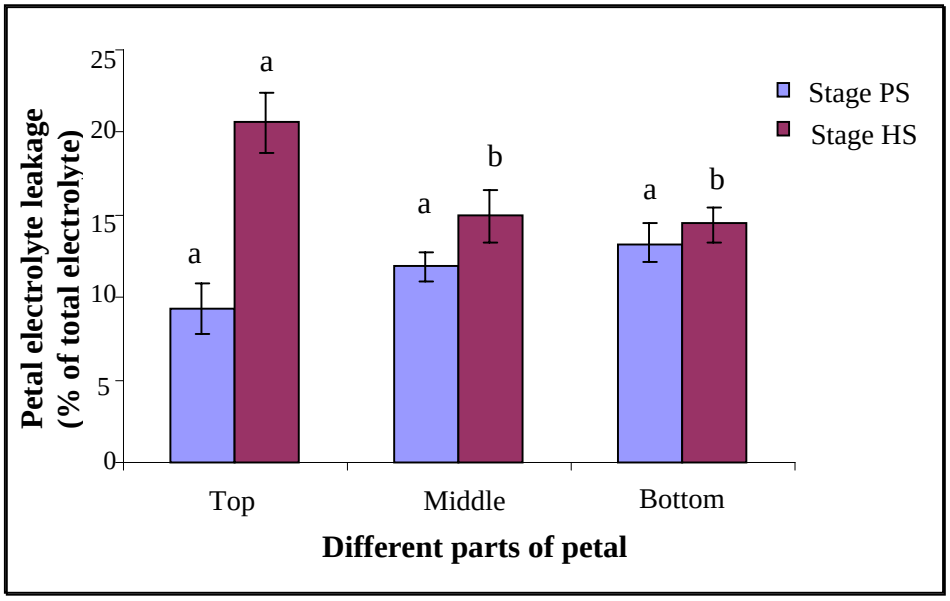


Figure 4. 2 Changes in electrolyte leakage from different parts of petunia petals

Pre-senescence (PS): part of the petal starting to drop; half senesced flower (HS): part of the petal edge curled up.

Petunia flowers from Stage PS and HS were separated into top, middle and bottom. In each treatment, there were five replicate flowers for determination of petal electrolyte leakage. Vertical bars represent means ± SE for three determinations from individual petal extracts. The bars with different letters at the same stage indicate that the results are significantly different using Tukey's method ($p<0.05$).

4.2.2 Effect of STS and ethanol on cut petunia flower senescence

4.2.2.1 Effect of STS and ethanol on fresh weight changes of petunia flowers

Petunia flowers were harvested at the open stage (Stage 7), pulsed with three different concentrations of STS for 24 hours and then transferred to water. About 7 days later, the fresh weight of water-treated (control) petunia flowers started to decrease (*Figure 4.3*). However, that of the cut petunia flowers pulsed with 0.3 mM STS remained at a similar level throughout the experiment (*Plate 4.3 B*).

There was a significant difference between the fresh weigh of flowers treated with 4% ethanol and control flowers on day 10 (*Figure 4.4 B*), while those treated with 6% of ethanol had a significantly higher fresh weight from day 7 (*Figure 4.4 C*). However, 2% of ethanol had no effect on the fresh weight changes of cut petunia flowers (*Figure 4.4 A*).

Visual vase life assessment clearly demonstrated the anti-senescence effect of both 0.3 mM STS and 6% ethanol as more control flowers became wilted when compared to the STS- and ethanol-treated flowers (*Plates 4.7* and *4.8*)

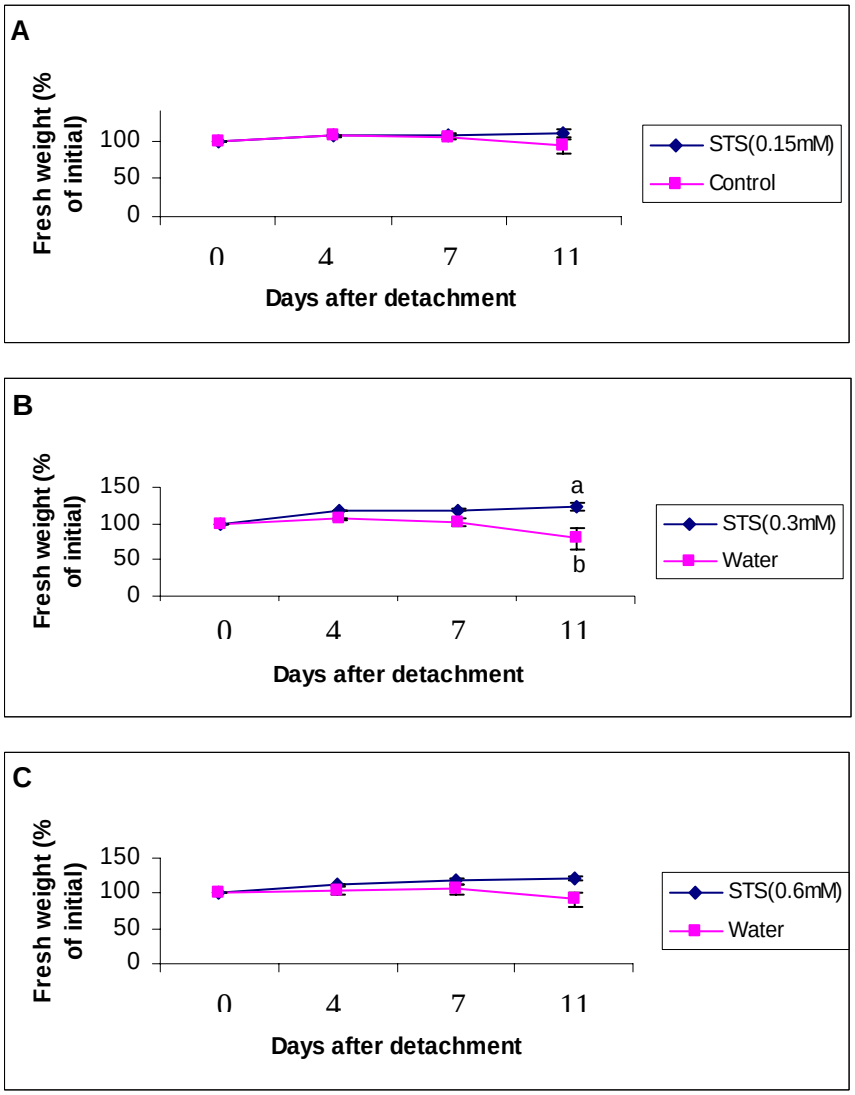


Figure 4. 3 Effect of different concentrations of STS on the fresh weight changes of detached petunia flowers

Detached open (Stage 7) petunia flowers were held in vials of distilled water or different STS solutions at room temperature for 24 hours, then transferred to water, and kept in a growth room. In each treatment, there were five replicate flowers. Vertical bars represent means ± SE. The bars with different letters indicate that the results are significantly different using Tukey's method ($p < 0.05$).

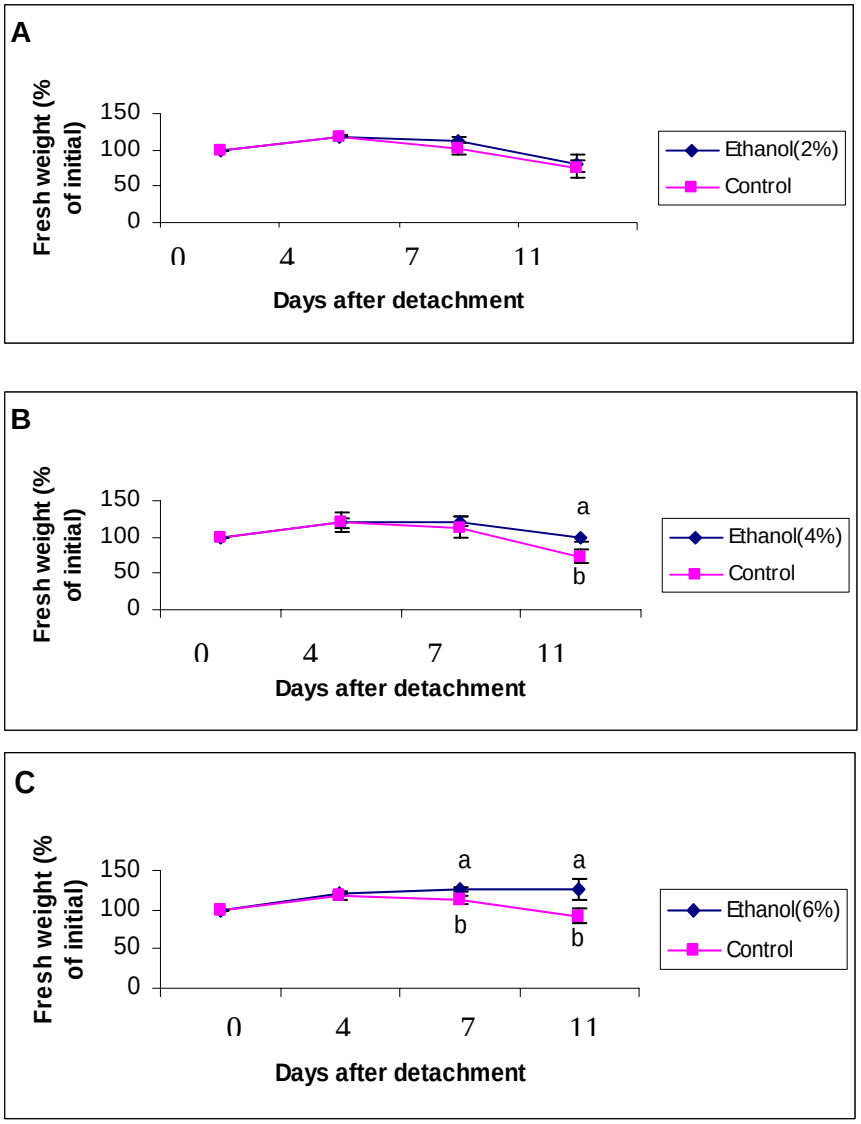


Figure 4. 4 Changes in the fresh weights of detached petunia flowers held in different concentrations of ethanol

Detached open (Stage 7) petunia flowers were held in vials of water or different ethanol solutions in a growth room. In each treatment, there were five replicate flowers. Vertical bars represent means ± SE. The bars with different letters indicate that the results are significantly different using Tukey's method ($p < 0.05$).

Plate 4. 7 Effect of STS treatment on senescence of petunia flowers

Detached open (Stage 7) flowers had been held in vials of 0.3 mM STS for 24 hours or distilled water before they were transferred to water and kept at room temperature. Photos were taken 10 days after the start of treatments.

Plate 4. 8 Effect of ethanol treatment on petunia flower senescence

Detached open (Stage 7) flowers were held in vials of distilled water or 6% ethanol in a growth room. The photo was taken two weeks after the start of the experiment.

4.2.2.2 Effect of STS and ethanol on ROS formation / accumulation

Since STS and ethanol delayed senescence of the petals of detached petunia flowers, their effects on ROS accumulation in isolated petunia petals were investigated. STS inhibited or delayed superoxide formation and accumulation in the isolated petunia petals since the control petals showed stronger purple coloration (more purple formazan staining) (*Plate 4.9, right*) than the STS-treated petunia petals (*Plate 4.9, left*).

The effect of ethanol on superoxide accumulation and the hydrogen peroxide production in isolated petunia petals were also tested (*Plate 4.10*). Ethanol application inhibited superoxide and hydrogen peroxide accumulation in isolated petunia petals as well.

STS (0.3 mM) Control

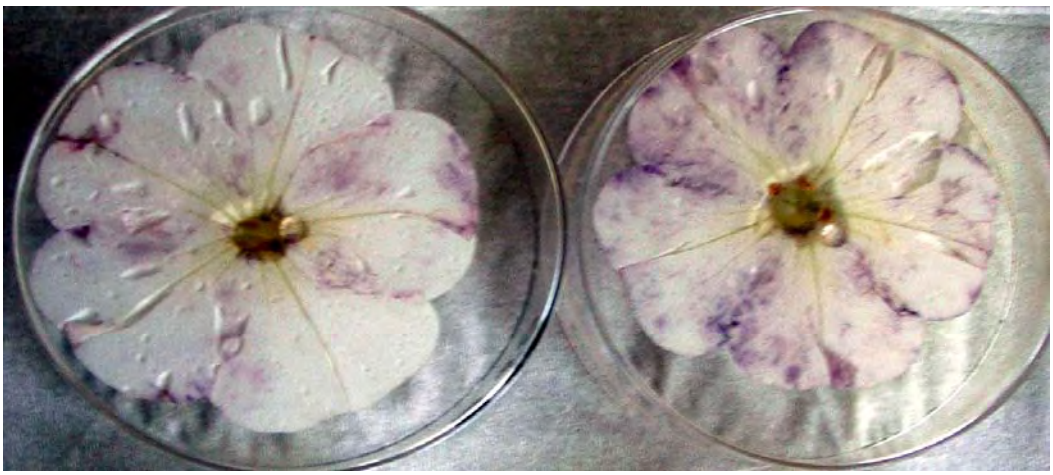


Plate 4. 9 Effect of STS on superoxide accumulation in isolated petunia petal

Detached open petunia flowers had been held in vials of distilled water (right side) or 0.3 mM STS solution (left side) for 24 hours at room temperature before they were transferred to distilled water and kept in a growth room for 5 days. After this, superoxide was detected following incubation of the petals for 12 hours in NBT (1%).

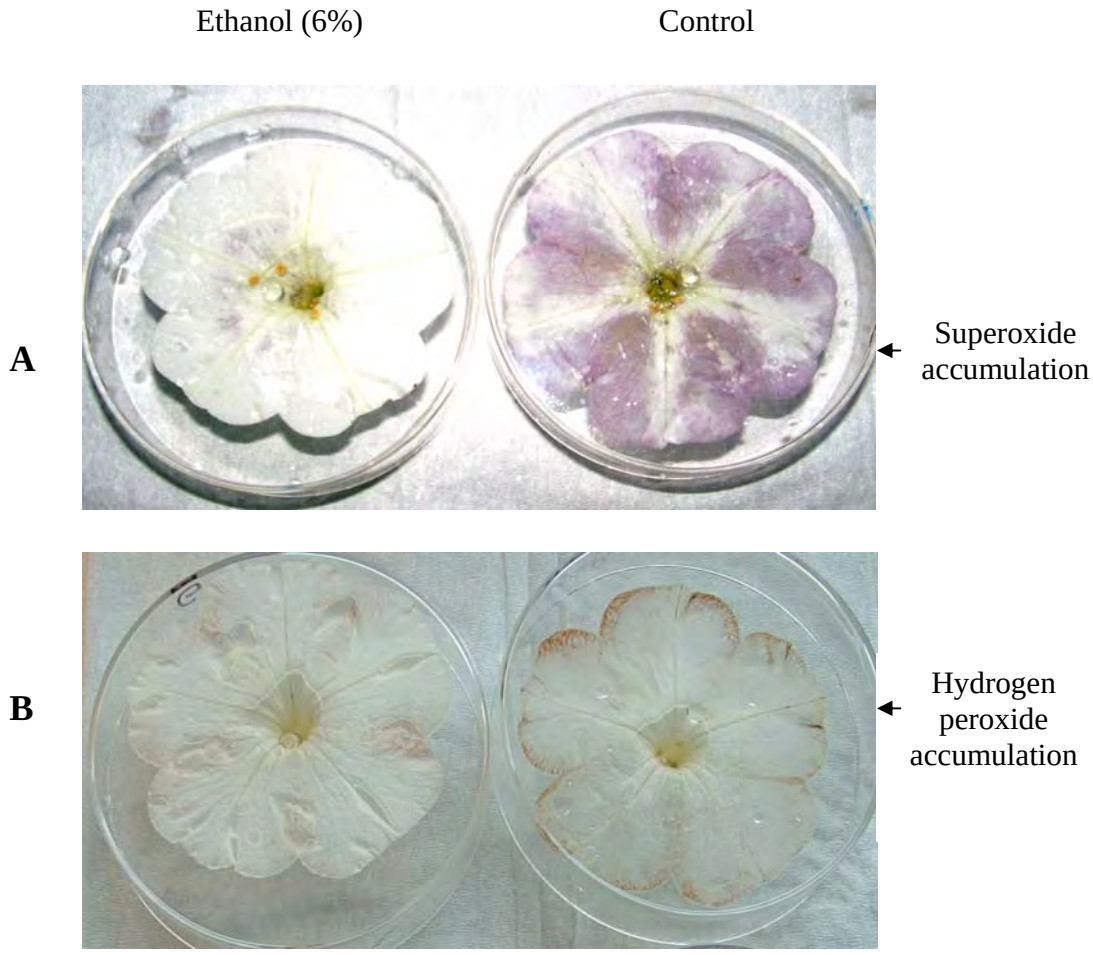


Plate 4. 10 Effect of ethanol on levels of reactive oxygen species in isolated petunia petals

Detached open petunia flowers had been held in vials of distilled water (right side) or 6% ethanol solution (left side) for 3 days before superoxide detection using NBT (1%) staining (A). After being in water or 6% ethanol for 2 days, they were transferred to water and kept in a growth room for four days before hydrogen peroxide accumulation was detected in 1% DAS (B).

4.2.2.3 Effect of ethanol on ovary weight and petal electrolyte leakage in petunia flowers

After 10 days in 6% (v/v) ethanol, senescence-related increase in petal electrolyte leakage and ovary weight were significantly retarded (*Figure 4.5 A* and *B*). Concomitantly, the petal weight was maintained in ethanol-treated flowers when compared to that of the control flowers (*Figure 4.5 C*).

4.2.3 Direct effect of ethanol on isolated petunia petals

Ethanol can delay petal senescence of a detached petunia flower consisting of corolla, androecium, gynoecium and peduncle. The interrelationships among these flower parts may involve and affect the rate of petal senescence. Therefore, the isolated petal system was used in order to assess the direct anti-senescence effect of ethanol in petunia.

The vase life of the isolated petals held in 6% ethanol was much better than that in water (*Plate 4.11*). The longevity of isolated petunia petals treated with 6% ethanol was nearly twice as long as when they were held in water (*Figure 4.6*).

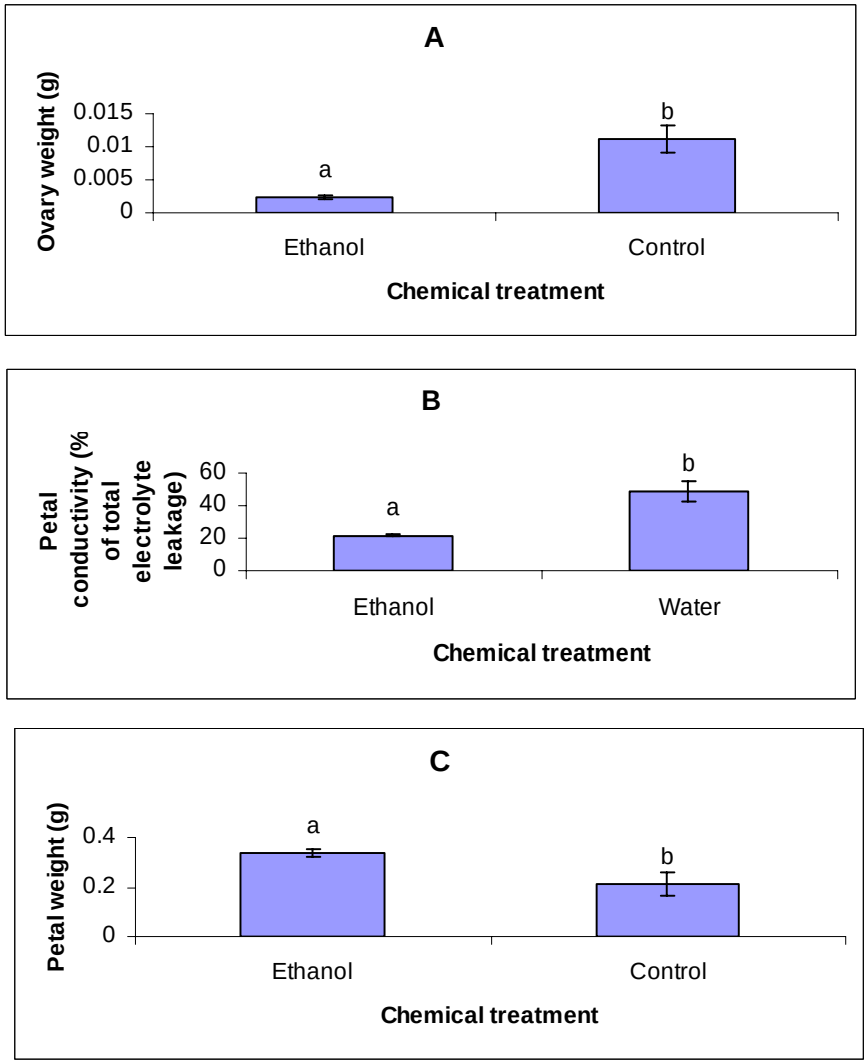


Figure 4. 5 Effect of ethanol treatment on A: ovary weight, B: petal conductivity (electrolyte leakage), C: petal weight of detached petunia flowers

Detached open flowers were held in vials of water (control) or 6% ethanol in a growth room for 10 days. In each treatment, there were three replicate flowers. Vertical bars represent means ± SE. The bars with different letters indicate that the results are significantly different using Tukey's method ($p < 0.01$).

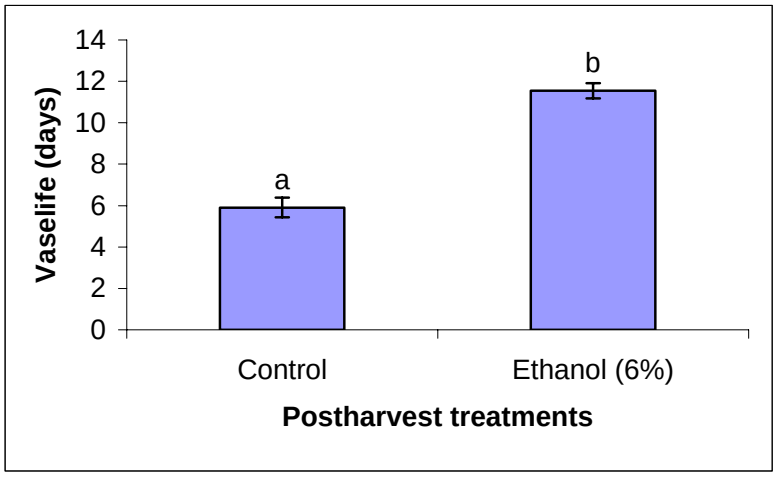


Figure 4. 6 Effect of ethanol treatment on the vase life of isolated petunia petals

Isolated open petunia petals were held in vials of water or 6% ethanol in a growth room for two weeks. In each treatment, there were five replicate flowers. Vertical bars represent means ± SE. The bars with different letters indicate that the results are significantly different using Tukey's method ($p<0.05$).

Plate 4. 11 Effect of ethanol treatment on senescence of isolated petunia petals

Isolated petunia petals from stage 7 or 8 flowers had been held in vials of distilled water (Rows 2 and 4) or 6% ethanol (Rows 1 and 3) in a growth room.

4.2.3.1 Effect of ethanol on fresh weight changes and electrolyte leakage of isolated petunia petals

The trends of changes in petal fresh weight and electrolyte leakage in response to ethanol treatment were also investigated in isolated petunia petals. Ethanol could obviously maintain petunia petal fresh weight during the experimental period (*Figure 4.7, upper graph*). There was a significant difference between the fresh weight of the ethanol and water-treated petunia petals from day 5. Furthermore, the electrolyte leakage of water-treated petunia petals increased significantly from day 3. However, that of petals held in 6% of ethanol was almost stable in the one-week experiment period (*Figure 4.7, lower graph*).

4.2.3.2 Effect of ethanol on the pigment contents of isolated petunia petals

There was a drop in the pigment contents by day 3 irregardless of the treatments (*Figures 4.8* and *4.9*). The contents of chlorophyll a and b were slightly higher in water-treated petunia petals than in the ethanol-treated ones on per fresh weight basis. Similarly, in the ethanol treatment the carotenoid level was a little lower when compared to the control petals particularly on day 7. However, the difference in pigment level was not significant.

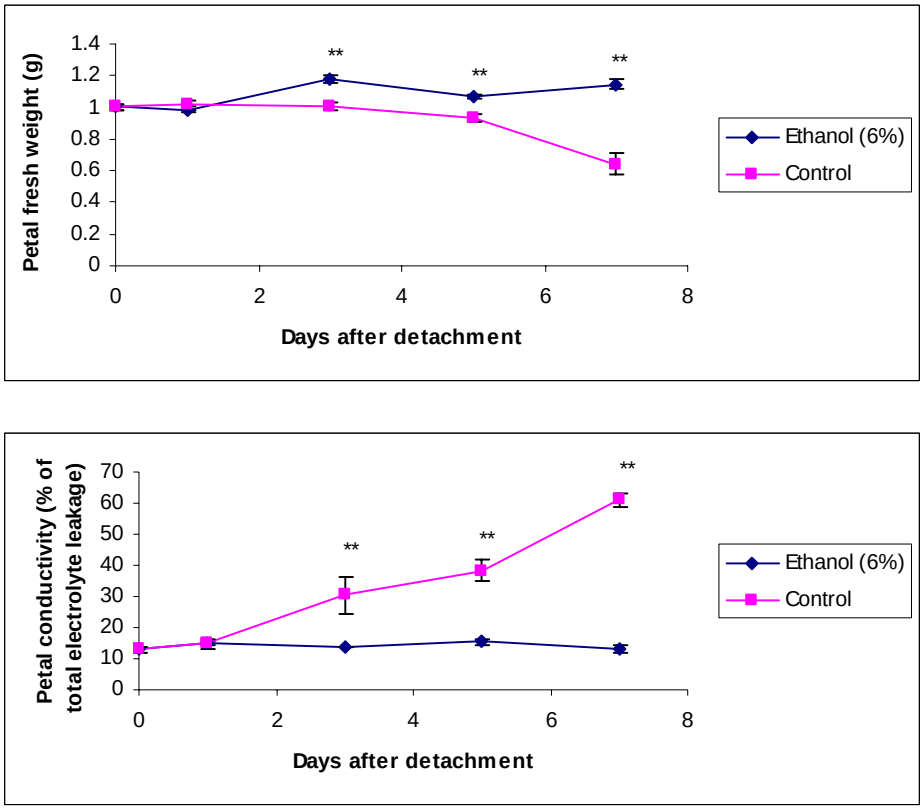


Figure 4. 7 Changes in the fresh weight (upper figure) and electrolyte leakage (lower figure) of isolated petunia petals held in 6% ethanol or water

Isolated petunia petals from open flowers were held in vials of distilled water or 6% ethanol in a growth room. In each treatment, there were three replicate flowers. Vertical bars represent means ± SE. The bars with ** indicate that the results are significantly different using Tukey's method ($p < 0.01$).

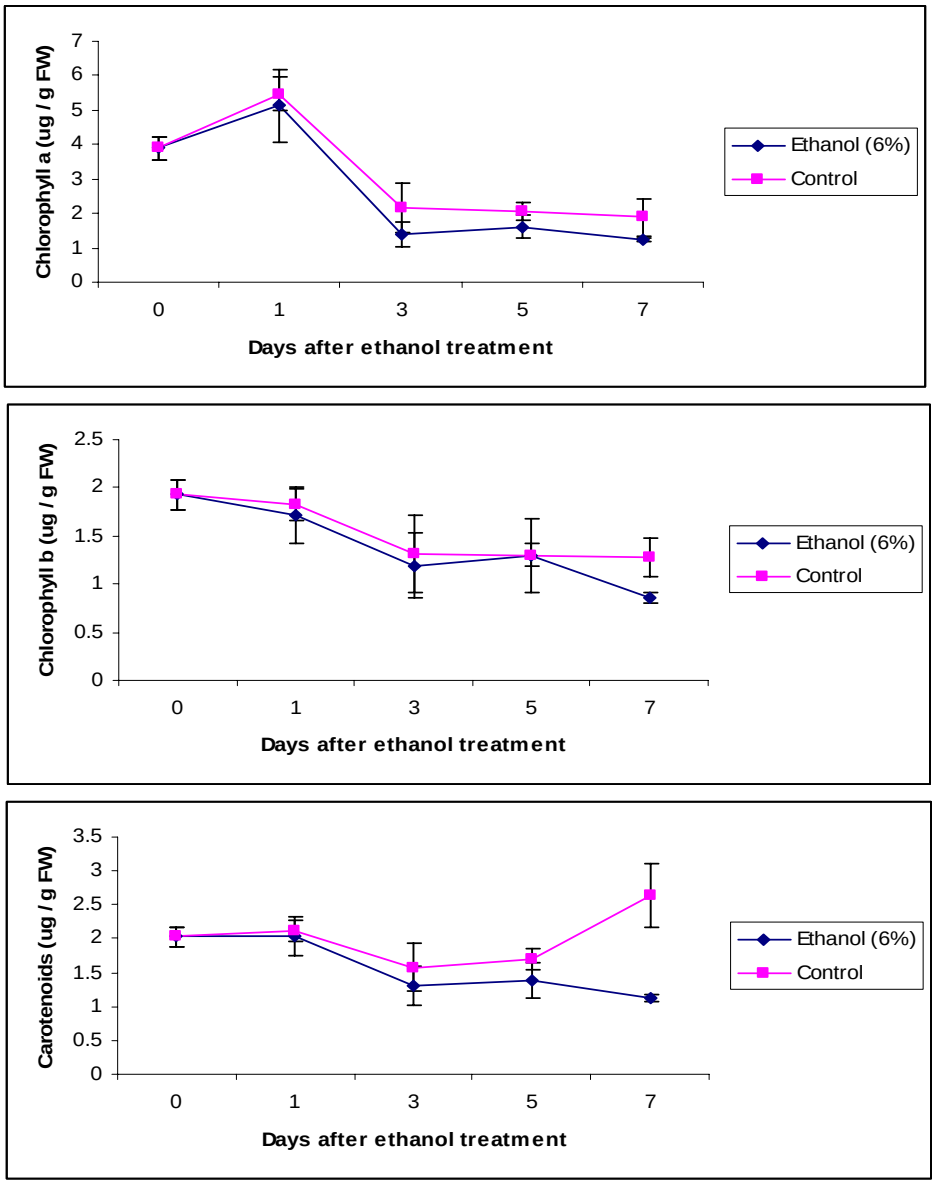


Figure 4. 8 Changes in the contents, on per fresh weight basis, of chlorophyll a, chlorophyll b and carotenoids in isolated petunia petals held in 6% ethanol or water

Isolated petals from open petunia flowers were held in vials of distilled water or 6% ethanol in a growth room. In each treatment, there were three replicate flowers. Vertical bars represent means ± SE.

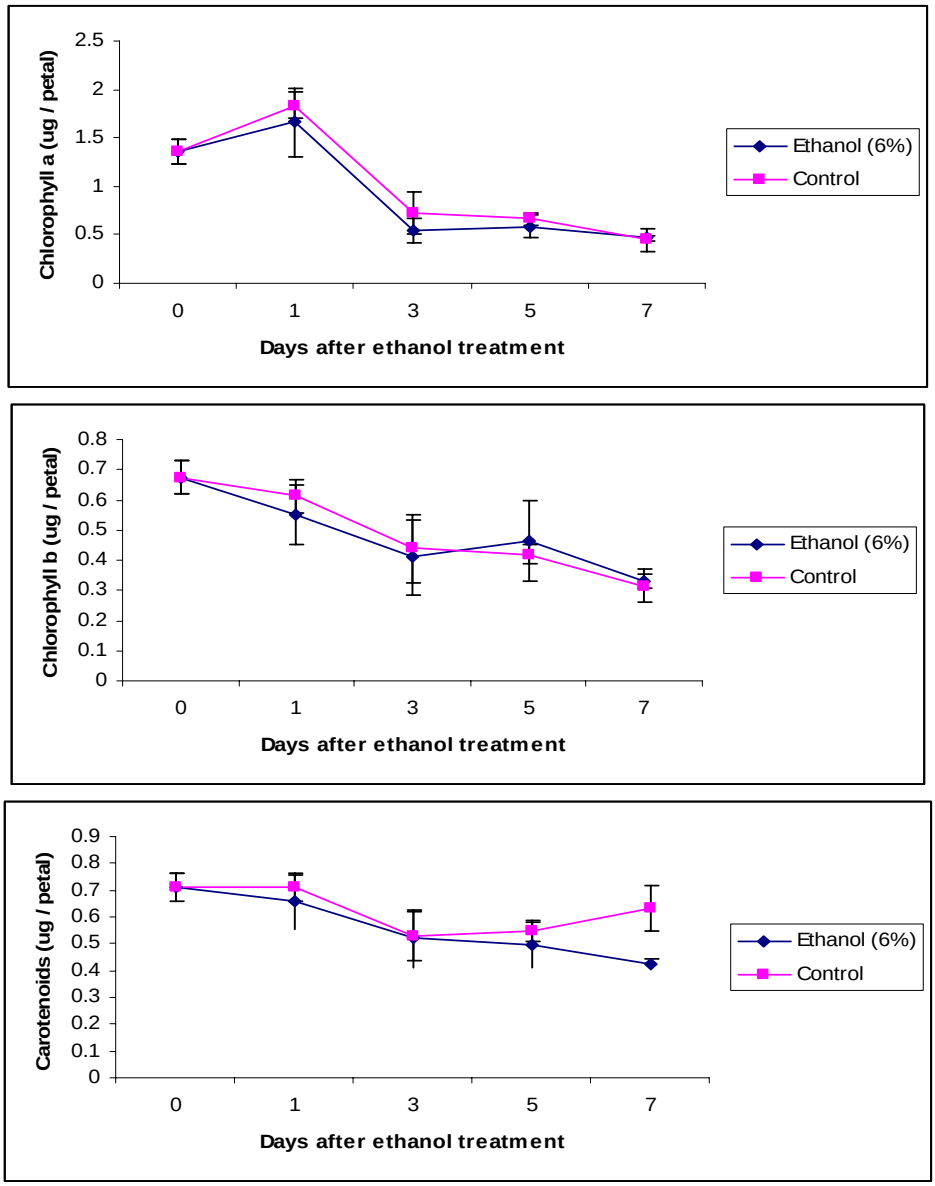


Figure 4. 9 Changes in the contents, on per petal basis, of chlorophyll a, chlorophyll b and carotenoids in isolated petunia petals held in 6% ethanol or distilled water

Isolated petals from open petunia flowers were held in vials of water or 6% ethanol in a growth room. In each treatment, there were three replicate flowers. Vertical bars represent means ± SE.

4.2.3.3 Changes of soluble proteins of petunia petals

Petunia flower development and senescence were associated with a general gradual loss of soluble protein bands after their separaion using one-dimensional SDS-PAGE and were then stained with Coomassie blue first (*Plate 4.12*, upper plate). Silver staining revealed more clearly the pattern of protein changes in petunia during petal development and senescence (*Plate 4.12*, lower plate). Four (arrowed) interesting bands were observed with their intensities increased or decreased during petal senescence.

When the protein level was expressed based on FW, the control petals had a slightly higher protein level on day 1 (*Figure 4.10*). Then the content of protein in both ethanol- and water-treated petunia petals decreased from day 1 to day 3. While this decrease in water-treated control flowers continued, protein levels in ethanol-treated petals remained higher. There was a significant difference in protein levels between ethanol- and water-treated petals. Ethanol-treated petals had significantly higher protein levels on day 3, 5 and 7 than water-treated petals.

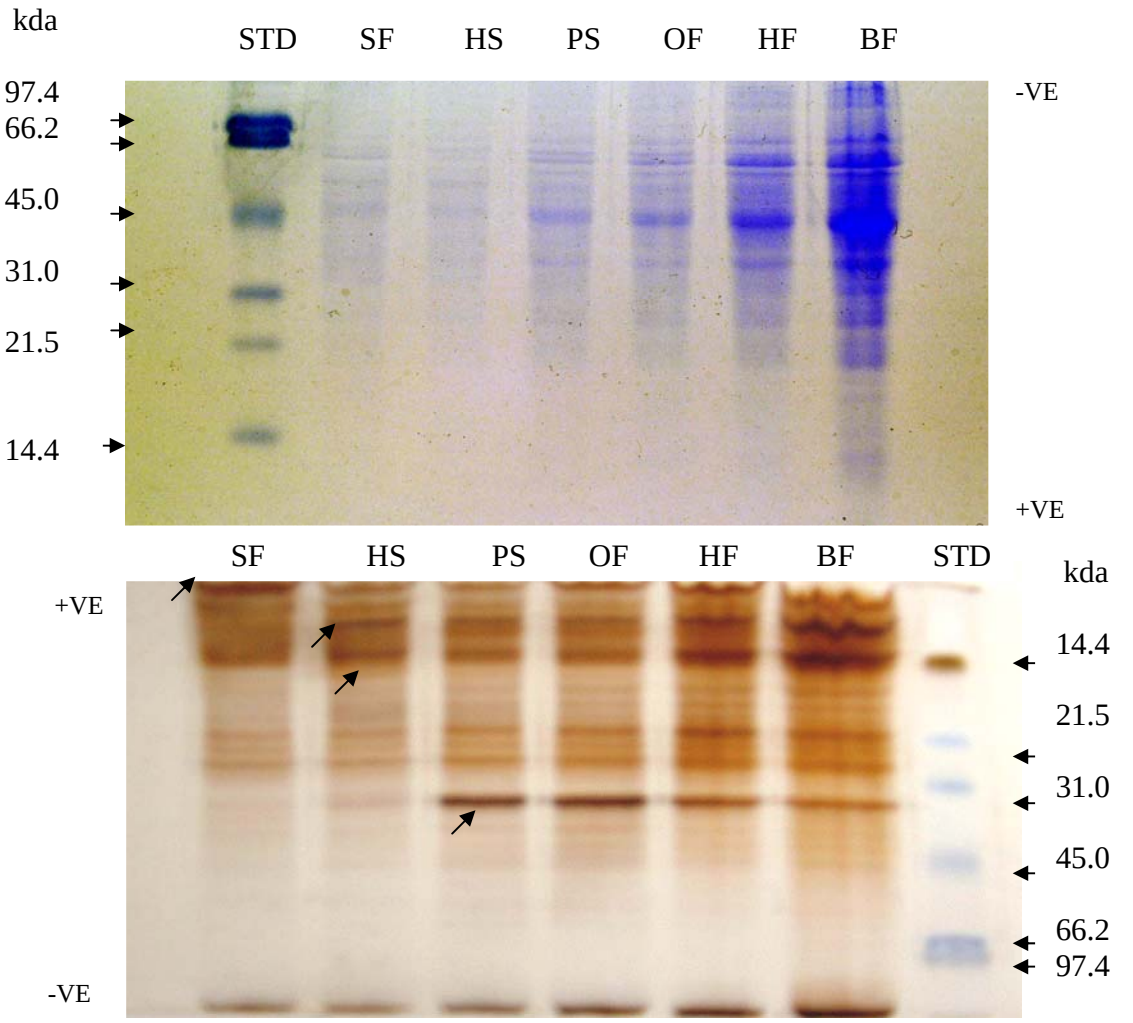


Plate 4.12 Commassie brilliant blue (upper) and silver staining (lower) of SDS-PAGE gels showing patterns of soluble proteins in petunia petals at different stages of development and senescence

Lanes 1-6: petal extracts from 6 developmental and senescence stages: bud flower (BF); half open flower (HF); open flower (OF); pre-senescence (PS): part of the petal starting to drop; half senesced flower (HS): part of the petal edge curled up; senesced flower (SF): part of the petal tissue appeared to be crispy. Arrows in the lower plate showed the four bands of interests. Lane 7: Low range SDS-PAGE Molecular Weight Standards (STD): phosphorylase b 97.4 kDa, bovine serum albumin 66.2 kDa, ovalbumin 45 kDa, carbonic anhydrase 31 kDa, soybean trypsin inhibitor 21.5 kDa, and lysozyme 14.4 kDa.

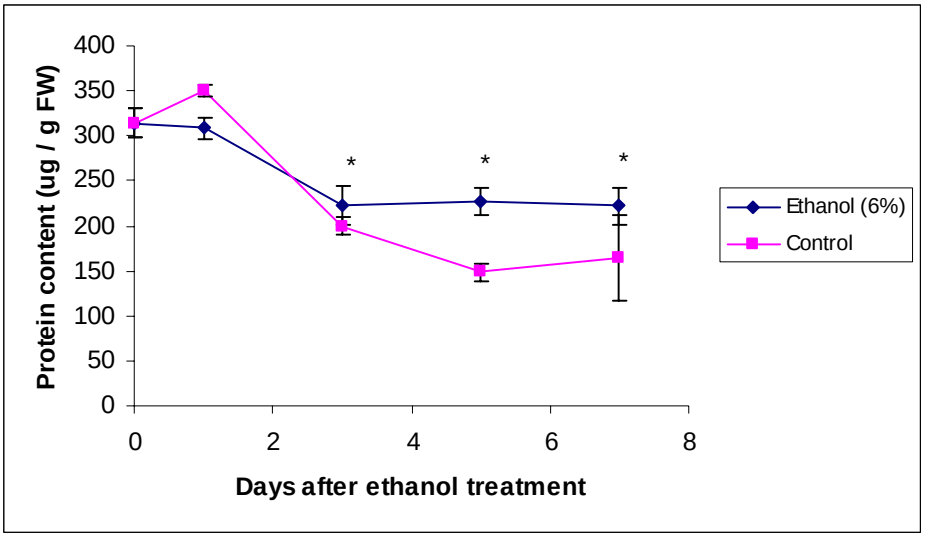


Figure 4. 10 Changes in the level of buffer-soluble proteins on per FW basis of isolated petunia petals held in 6% ethanol or water

Isolated petals from Stage 7 open flowers were held in vials of distilled water or 6% ethanol in a growth room. In each treatment, there were three replicate flowers. Vertical bars represent means ± SE. The bars with * indicate that the results are significantly different using Tukey's method ($p < 0.05$).

4.2.3.4 Effect of ethanol on antioxidative enzyme activities in isolated petunia petals

The activities and isozymes of three antioxidative enzymes in petunia petals were analysed in order to assess if they were influenced by ethanol treatment. Also in an attempt to determine the best way to express changes in enzyme activity during flower development and senescence, the enzyme data had been calculated based on three

different parameters, i.e. per petal, per milligram soluble protein in petal extracts and per gram fresh weight of petal tissues. The following results showed that the anti-senescence effect of ethanol on petunia petals was associated with the retardation of a decrease in antioxidative enzymes, SOD, AP and POD, during senescence.

4.2.3.4.1 Determination of SOD activity

Following treatment of isolated petunia petals with ethanol or water for various times, SOD activity in the petals were determined. The ethanol-treated petals had a significantly higher level of SOD activity than that of the control petals from day 8 whether it was expressed based on per petal (*Figure 4.11A*) or per gram (*Figure 4.11B*) of petal fresh weight. The ratio of SOD activity between ethanol- and water-treated petals also showed a significant increase on day 8. This indicates a higher level of SOD activity in ethanol-treated petals than that of the control petals (*Figure 4.12*).

4.2.3.4.2 Determination of POD activity

The total POD activity in isolated petunia petals was almost stable during the first 8 days of treatment with ethanol or water (*Figure 4. 13 A*). However, on day 10 the ratio of POD activity between ethanol-treated and control petals was significantly increased when the enzyme activity was expressed on a per petal basis (*Figure 4. 13 B*).

4.2.3.4.3 Determination of AP activity

A similar trend was found in the changes of cytoplasmic AP activity, with a little change between the control and ethanol-treated petals from day 1 to 8. On day 10, there was a significant increase in the ratio of AP activity between ethanol treatment and water

control (*Figure 4.14*). Therefore, AP activity was higher in ethanol-treated petals than that of water control.

4.2.3.5 Effect of ethanol on antioxidative isozymes in isolated petunia petals

Isolated petunia petals treated with ethanol or water were harvested at different days for isozyme analysis. Three SOD isoforms were observed in the extracts of control and ethanol-treated petunia petals in the first 5 days from the start of the experiment (*Plate 4.13 A*). The ethanol-treated petals had a new isozyme band (arrowed), which became just detectable. The intensities of the three major isozymes in ethanol-treated petals seem slightly higher than those of the control petals at day 7 (*Plate 4.13 A).* Replicate analysis (*Plate 4.13 B)* revealed that the new isozyme induced in the ethanol-treated petunia petals on day 7 was not stable. On the contrary, two extra minor bands (arrowed) appeared in the water-treated petals. Moreover, the difference in staining intensities among the major bands of S_1 and S_4 in ethanol- or water-treated petals was not greatly obvious regardless of same fresh weight or tissue load in each well. Then the profiles of SOD isozymes between control and ethanol-treated petals harvested on day 10 were compared. A very clear difference in the three main isozyme bands, S_1, S_2 and S_4 based on equal tissue load (per lane) for denaturing gel electrophoresis, was found between the control and ethanol-treated petunia petals (*Plate 4.13 C*). Ethanol-treated petunia petals had stronger isozyme activities including S_1 (Mn-SOD), S_2 and S_4 (Fe-SOD) (arrowed) when compared to the control petals.

About POD, very little difference was found in the intensities of all isozyme bands from day 1 to day 3. However, the intensity of P_3 (arrowed) was increased more on day 5 and 7 in control than in the ethanol treatment (*Plate 14 A*). In the repeat experiment, this was confirmed (*Plate 14 B*) in both equal tissue and equal weight loading of day 7 petal extracts. Moreover, the intensity of P_1 isozyme band (arrowed) was also increased more in the control than in the ethanol treatment while that of P_6 band (arrowed) was reduced

in the control compared with that in the ethanol treatment at the same stage. Enzyme assays showed that on day 10 there was no significant difference in the ratio of POD activity of ethanol and water treated petals based on per fresh weight. However, the difference of POD activity based on per petal tissue was significant. The gel system (*Plate 14 C*) also gave some evidence that the staining intensities of P_6, and P_7 decreased more in the control than those in the ethanol-treated petals based on same tissue load of day 10 petal extracts. At the same time, the activity of the senescence-related band P_3 isoform in ethanol-treated petals was similar to that of the control petals.

About AP, the staining intensity of the main isoform, A_3, increased on day 3 in both the ethanol- and water-treated petunia petals (*Plate 4.15 A*). The intensity of A_3 isoform decreased in the control petals from day 5. However, the activities of AP in ethanol-treated petals decreased later. In the replicate experiment, similar results were also obtained with the extracts of petunia petals after ethanol treatment for 3 and 10 days. At day 10, the activities of A_1, A_3 and A_4 were higher in ethanol-treated petunia petals than those in the control petals (*Plate 4.15 B*).

4.2.3.6 Effect of ethanol on ROS formation in isolated petunia petals

The results that ethanol could inhibit superoxide and hydrogen peroxide accumulation in detached petunia flowers treated with 6% ethanol were confirmed again using the isolated petunia petal system. Isolated petunia petals from flowers at Stage 7 or 8 were treated with 6% ethanol or water for 3-5 days and then stained for 10 hours in NBT or 18-36 hours in DAS solutions, respectively. Hydrogen peroxide accumulation was detected in the control petals, particularly at the petal edge. This was expected to senesce earlier than the other parts of the petal tissue (*Plate 4.16 A*). However, the ethanol-treated petals were still healthy, and hydrogen peroxide was not detectable under the present experimental conditions. When an ethanol-treated petal showed visual staining at the edge, the control petal showed more intense staining, with the majority of the staining being associated with the netted vein system here and there (*Plate 4.16 B*).

Isolated petunia petals were also stained for superoxide accumulation with NBT. The control petals clearly showed more intense purple formazan staining than the ethanol-treated petals, with the majority of the staining being associated with the non-vein region (*Plate 4.16*).

4.2.3.7 Effect of ethanol treatment on ascorbate content in isolated petunia petals

There was little difference in the levels of ASC between ethanol- and water-treated petals in the first 5 days (*Figure 4.15*). On day 7, if the ascorbate content was expressed based on per petal, ethanol-treated petals had a slightly higher level of ascorbate than that of the control petals. However, the difference was not significant (*Figure 4.15 B*).

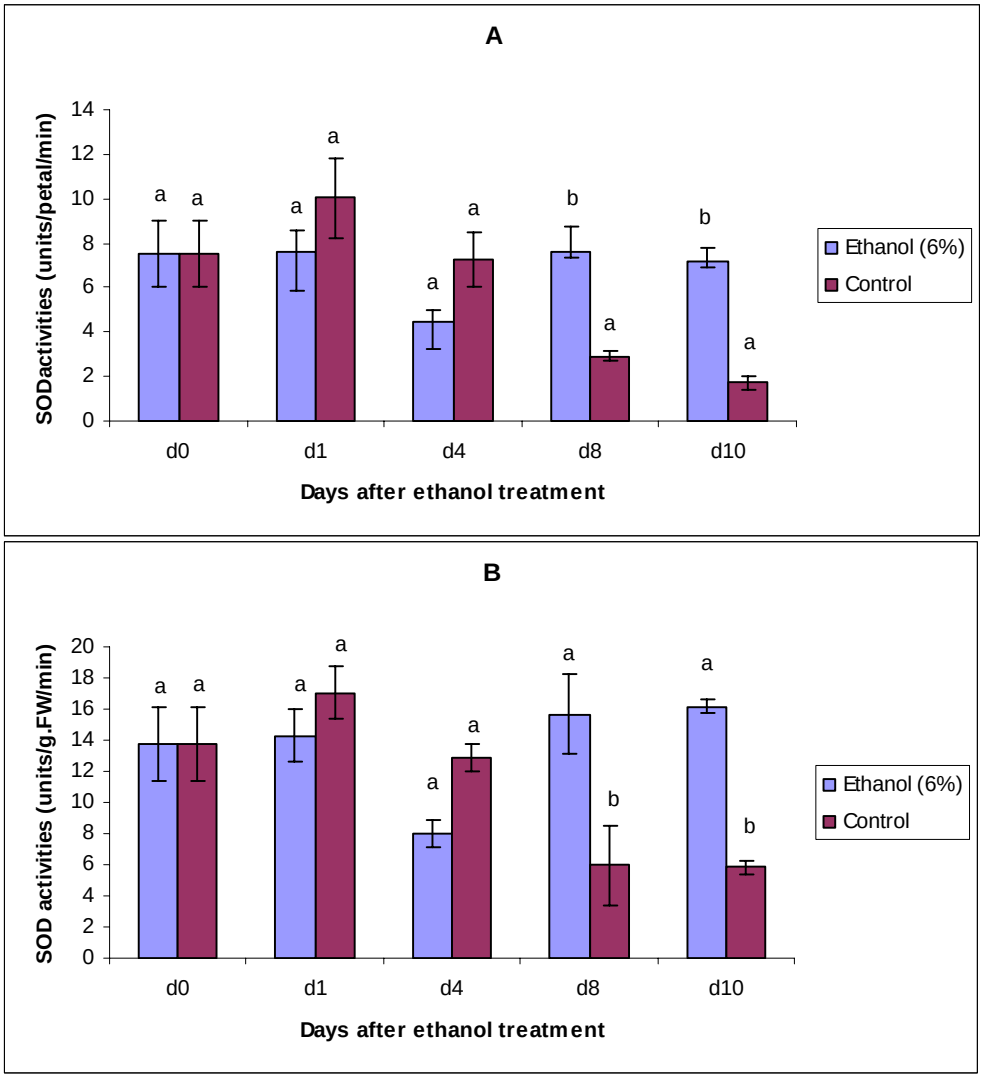


Figure 4. 11 Effect of ethanol treatment on SOD activity of isolated petunia petals. SOD activity was expressed in A: per petal; B: per g FW.

Isolated petals from Stage 6 open petunia flowers were held in vials of distilled water or 6% ethanol in a growth room. In each treatment, there were three replicate flowers. Vertical bars represent means ± SE. The bars with different letters indicate that the results are significantly different using Tukey's method ($p < 0.05$).

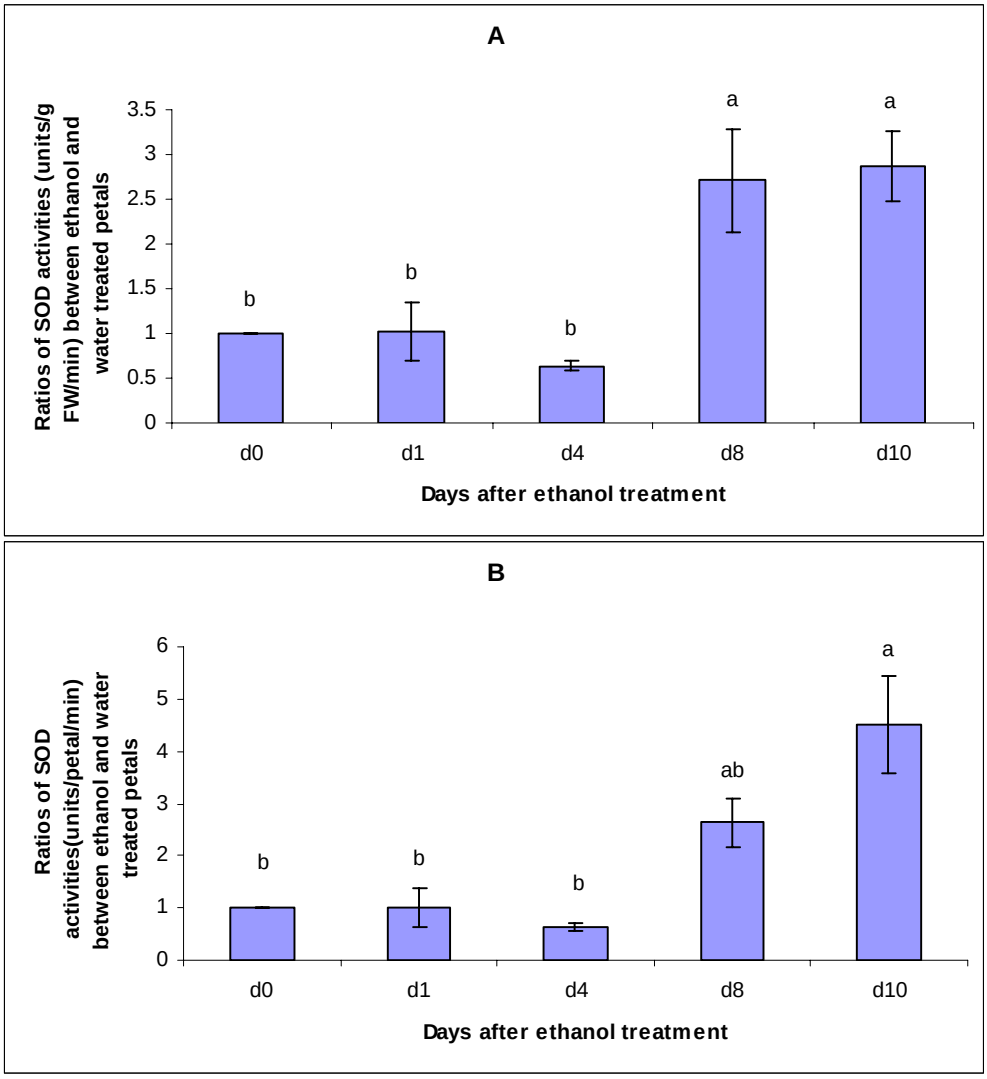


Figure 4. 12 Effect of ethanol treatment on SOD activity: SOD activity in ethanol-treated petals relative to that in water-treated petals. SOD activity was expressed in A: per g FW; B: per petal.

Isolated petals from open petunia flowers were held in vials of distilled water or 6% ethanol in a growth room. In each treatment, there were three replicate flowers. Vertical bars represent means ± SE. The bars with different letters indicate that the results are significantly different using Tukey's method ($p < 0.05$).

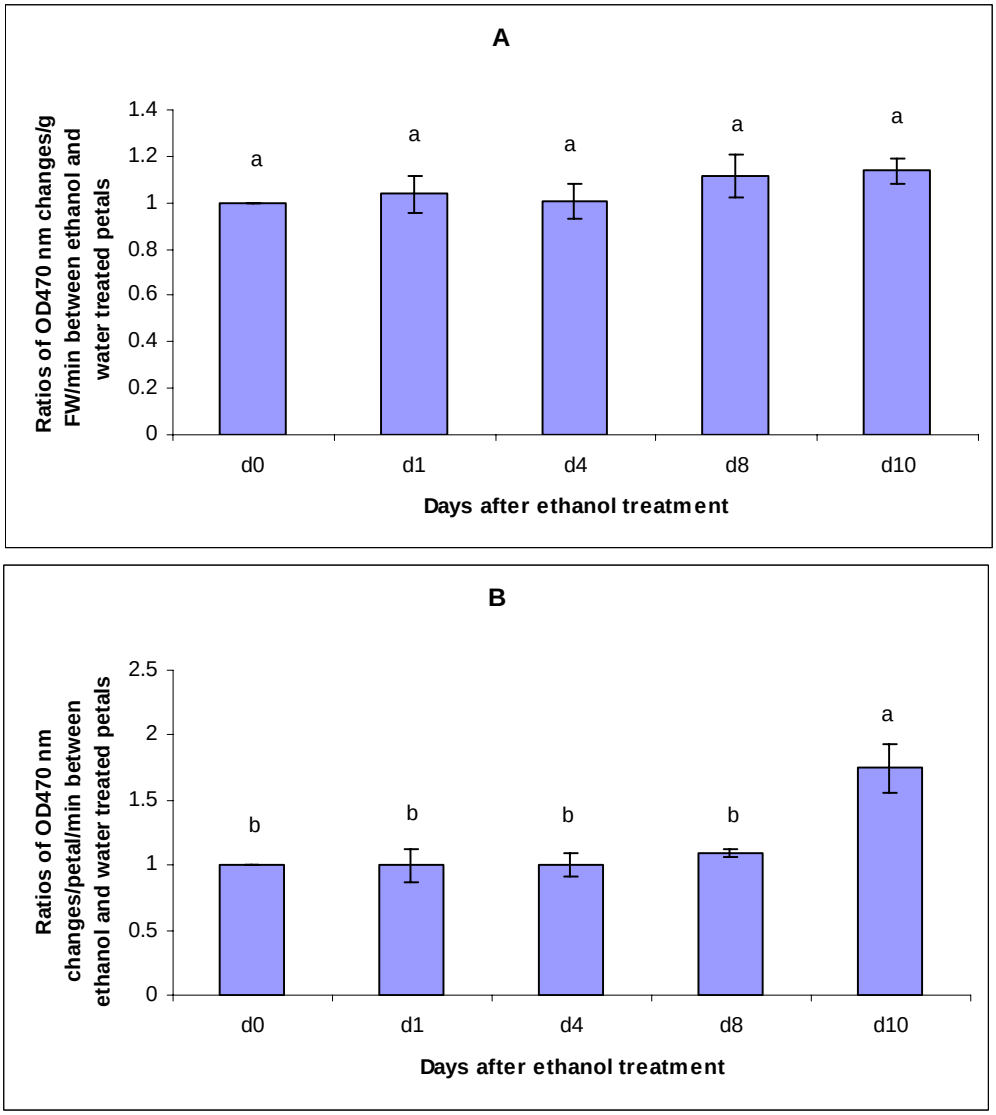


Figure 4. 13 Effect of ethanol treatment on POD activity: POD activity in ethanol-treated petals relative to that in water-treated petals. POD activity was expressed in A: per g FW; B: per petal basis.

Isolated petals from open flowers were held in vials of water or 6% ethanol in a growth room. In each treatment, there were three replicate flowers. Vertical bars represent means ± SE. The bars with different letters indicate that the results are significantly different using Tukey's method ($p < 0.05$).

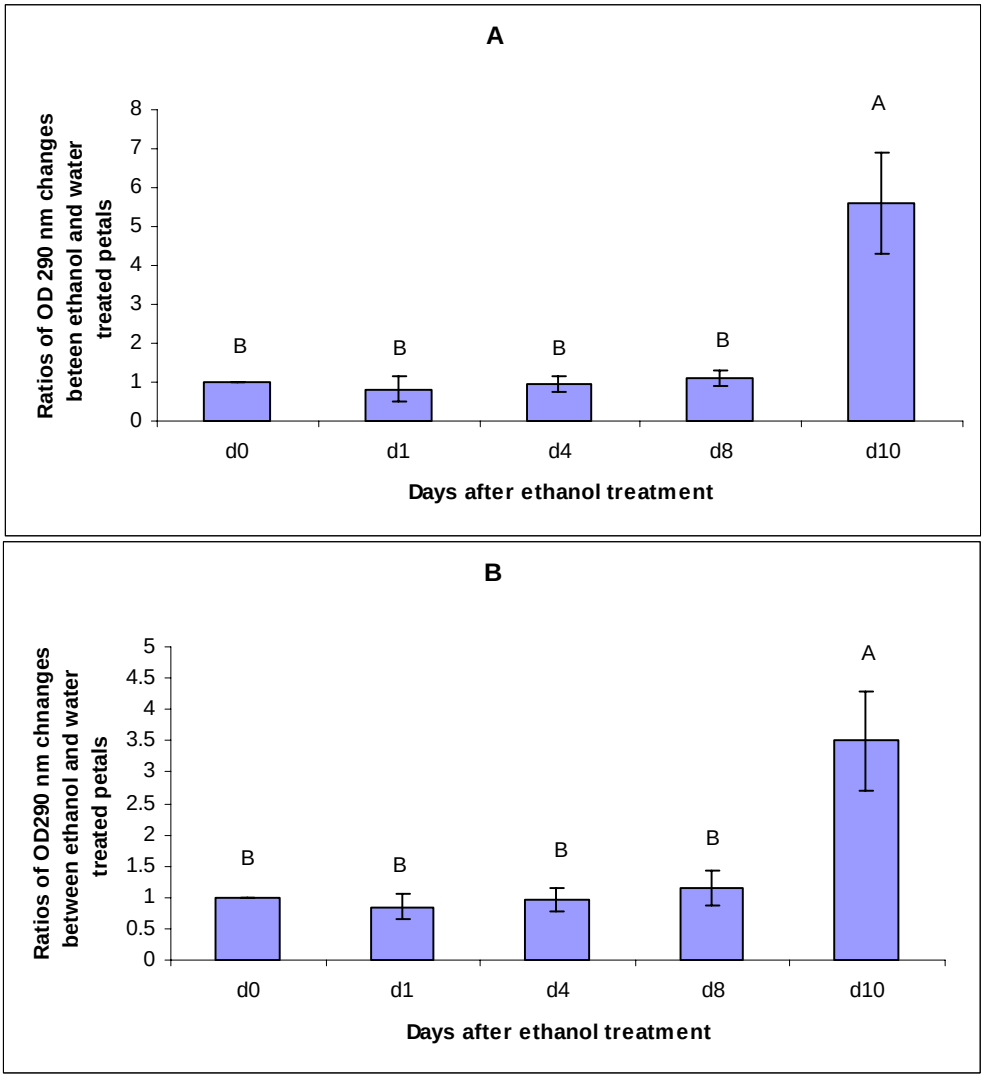


Figure 4. 14 Effect of ethanol treatment on AP activity: AP activity in ethanol-treated petals relative to that in water-treated petals. AP activity was expressed in A: per g FW; B: per petal.

Isolated petals from Stage 6 open petunia flowers were held in vials of distilled water or 6% ethanol in a growth room. In each treatment, there were three replicate flowers. Vertical bars represent means ± SE. The bars with different letters indicate that the results are significantly different using Tukey's method ($P<0.05$).

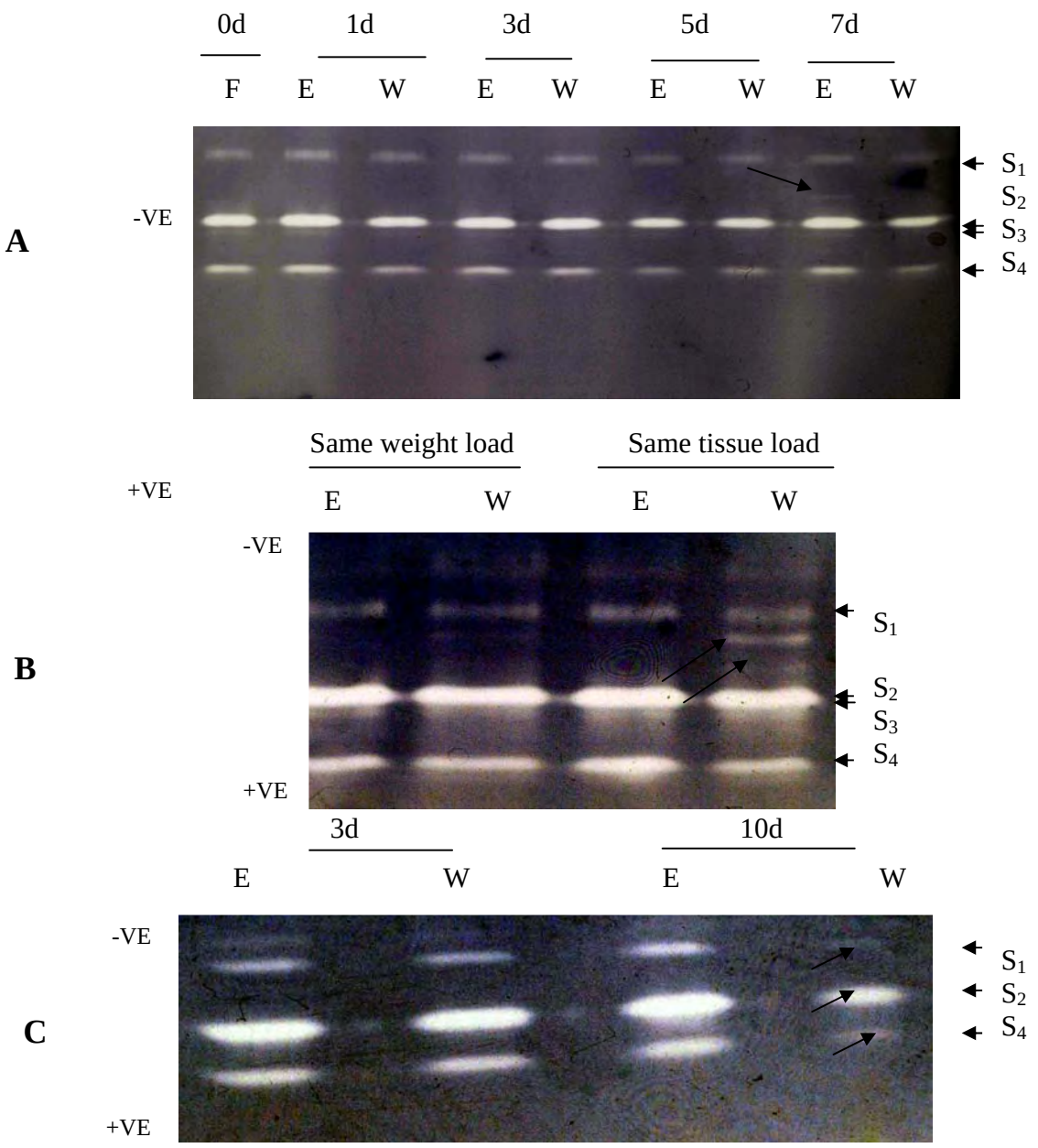


Plate 4. 13 Effect of ethanol treatment on SOD isoforms in extracts of isolated petunia petals

A: Treatment with ethanol (6%) or distilled water for 7 days; B: Effect of enzyme loading, per same weight or per same amount of tissue extracts of petals after 7 days of treatment; C: Same tissue load. (F: fresh petals without any treatment; E and W: represent ethanol and water treatments, respectively).

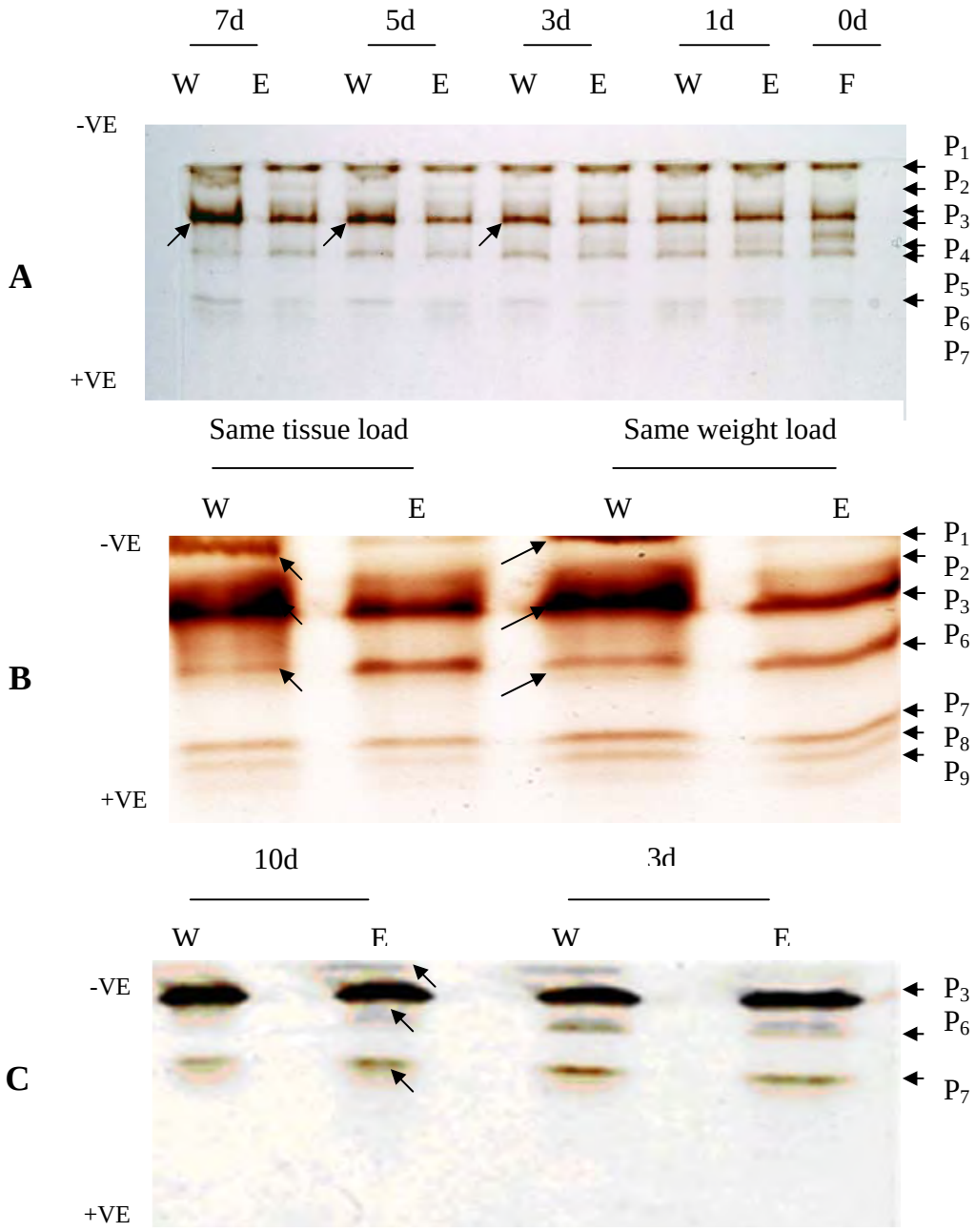


Plate 4. 14 Effect of ethanol treatment on peroxidase isoforms in extracts of isolated petunia petals

A: Changes in POD isoforms over 7 days of treatment with ethanol (6%) or distilled water; B: Effect of enzyme loading, per same weight or per same amount of tissue extracts of petals after 7 days of treatment; C: Same tissue load. (F: fresh petals without any treatment; E and W: represent ethanol and water treatments, respectively).

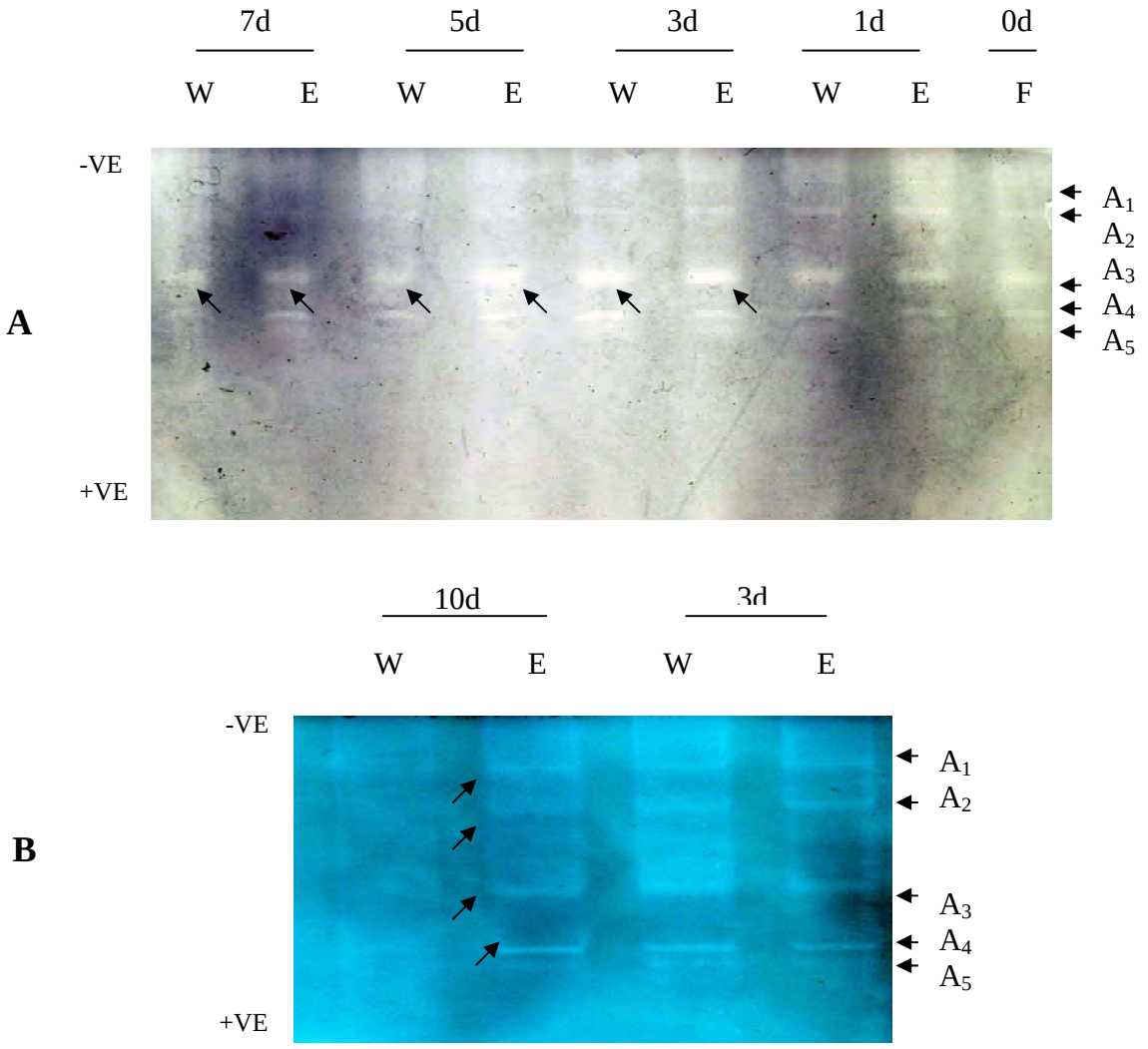


Plate 4. 15 Effect of ethanol treatment on ascorbate peroxidase (AP) isoforms in extracts of isolated petunia petal

A: changes in AP isoforms over 7 days of treatment with ethanol (6%) or distilled water. B: same tissue load. (F: fresh petals without any treatment; E and W: represent ethanol and water treatments, respectively).

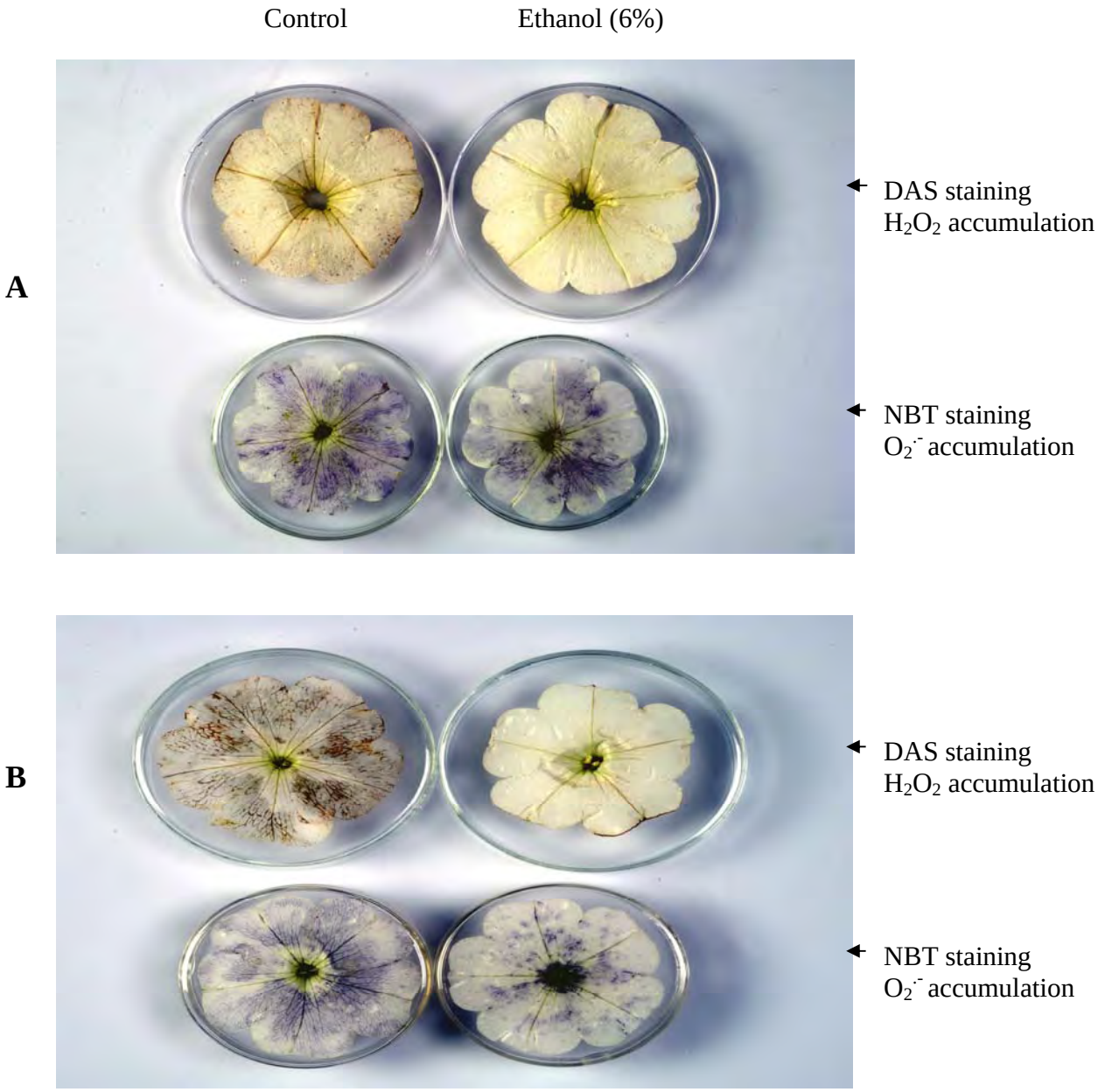
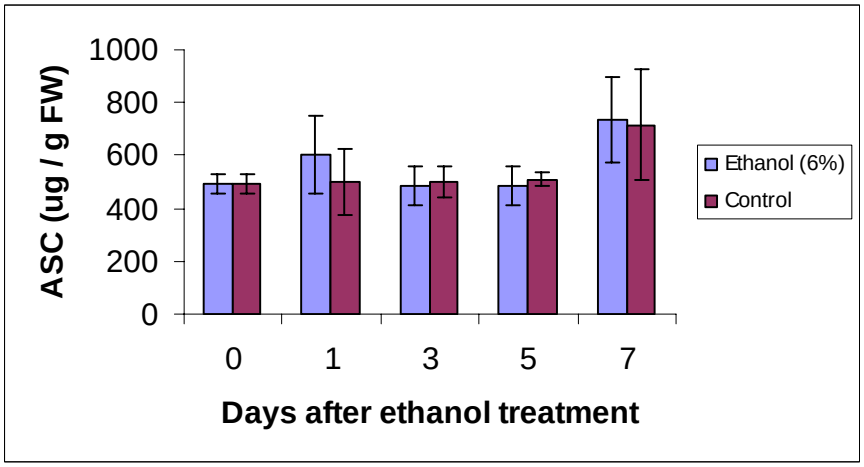


Plate 4. 16 Effect of ethanol treatment on superoxide and hydrogen peroxide accumulation in isolated petunia petals

Isolated petunia petals from stage 7 or 8 flowers were treated with 6% ethanol or distilled water for 3-5 days before they were stained for 10 hours in the NBT or 18-36 hours in DAS solutions, respectively. Plates A and B were from two repeat experiments.

A



B

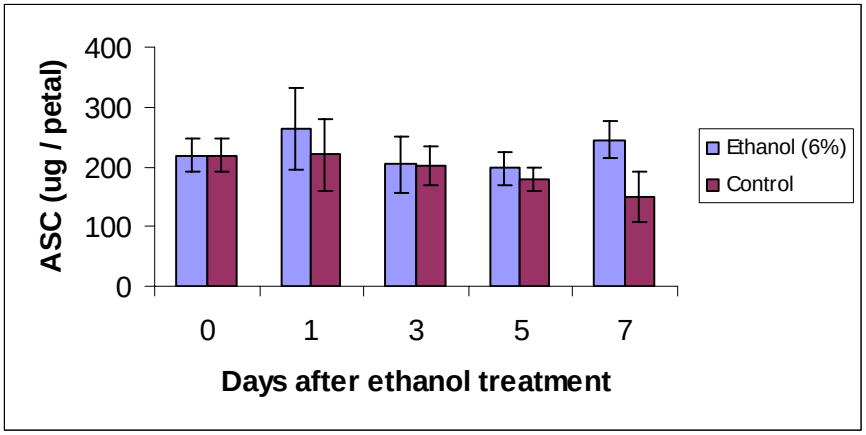


Figure 4. 15 Effect of ethanol treatment on the ascorbate content in isolated petunia petals. The results were expressed in A: per g FW and B: per petal.

Isolated open (Stage 7) petunia petals were held in vials of distilled water or 6% ethanol in a growth room. In each treatment, there were three replicate flowers. Vertical bars represent means ± SE.

4.2.4 Effect of ethanol on Cu-Zn SOD gene expression in isolated petunia petals

4.2.4.1 Evaluation of isolated RNA

The quality of total RNA isolated in this study was evaluated by absorbance ratio of 260 / 280, which was generally between 1.8 and 2.0 in DEPC-treated water. The integrity of RNA was checked by agarose gel electrophoresis. Two major 18S and 28S ribosomal RNA bands and many minor detectable mRNA bands below them were observed (*Plate 4.17*). When the quantity of RNA yield was studied, it was found that in water-treated petunia petals the RNA levels significantly decreased on days 3 and 6 when compared to day 0 (*Figure 4.16*). That represented a decrease of about 43% and 76% respectively on per petal basis ($p<0.001$). Further investigation revealed that the ratio of RNA content between ethanol- and water-treated petals did not change too much during the first three days, remaining to about one between day 3 and day 0 (*Figure 4.17*). However, that ratio increased to about 1.95 ($p<0.05$) at day 6, which was significantly higher than that between day 3 and day 0. This suggested that ethanol treatment significantly retarded the loss of RNA in petunia petals at later stages of petal senescence.

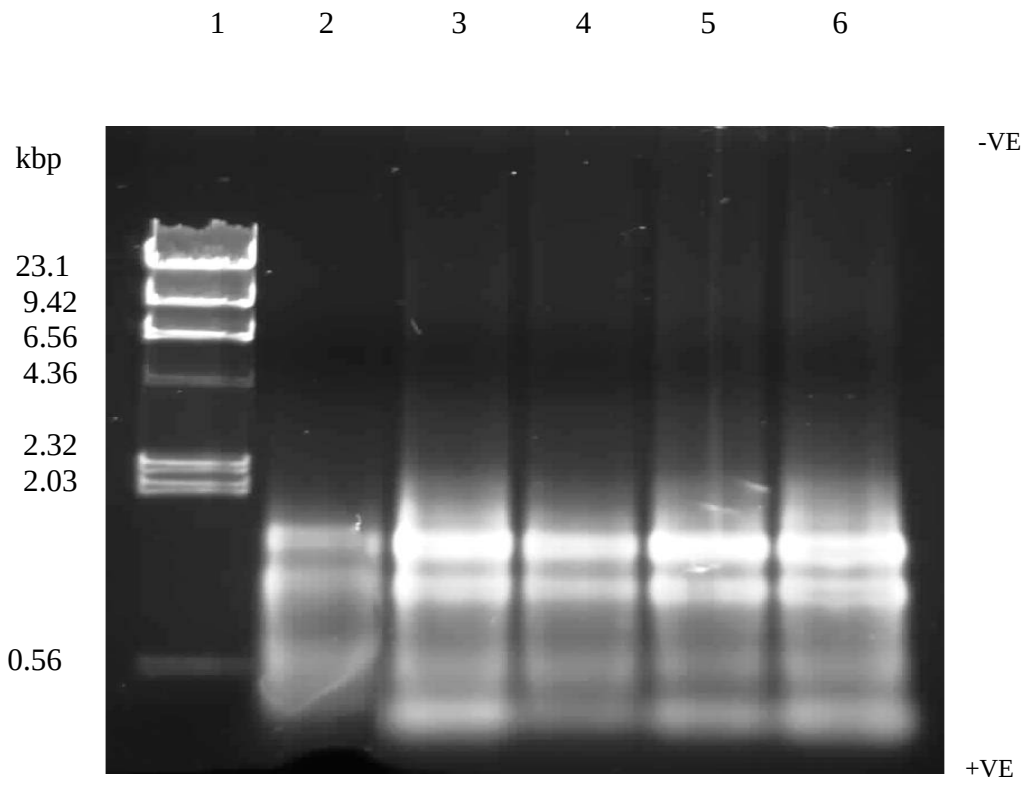


Plate 4. 17 Agarose gel electrophoresis of total RNAs isolated from petunia petals

Isolated petunia petals after different treatments for different days were subjected to agarose (1%) gel electrophoresis (80 V for 1 hour).

Lane 1: DNA markers (5 µl loaded); Lane 2: water treatment on day 6; Lane 3: ethanol treatment on day 6; Lane 4: water treatment on day 3; Lane 5: ethanol treatment on day 3; Lane 6: fresh petunia petals.

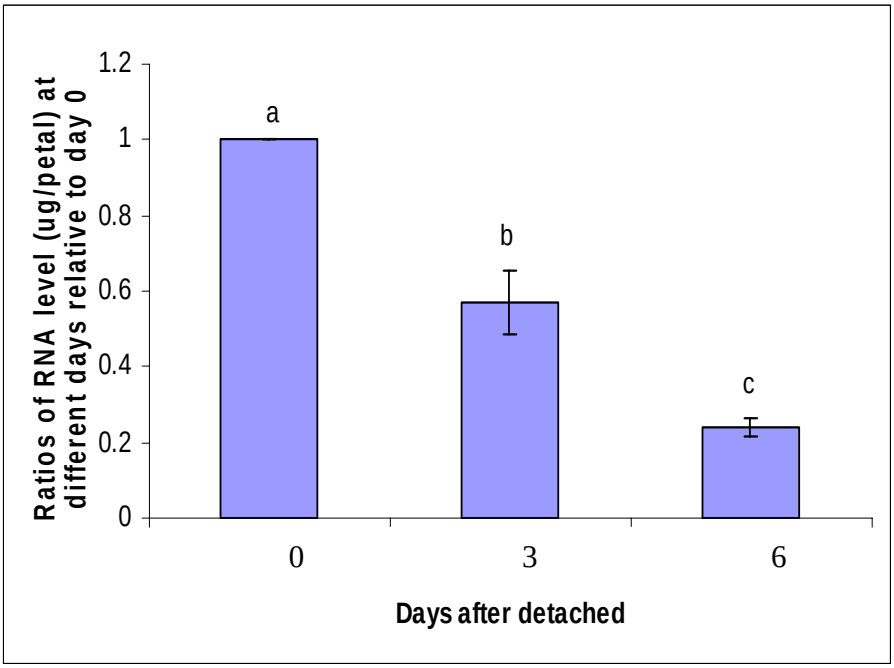


Figure 4. 16 Changes in total RNA content in water-treated petunia petals

Detached petals from open petunia flowers were held in vials of distilled water in a growth room. Vertical bars represent means ± SE. The bars with different letters indicate that the results are significantly different using Tukey's method ($p < 0.001$).

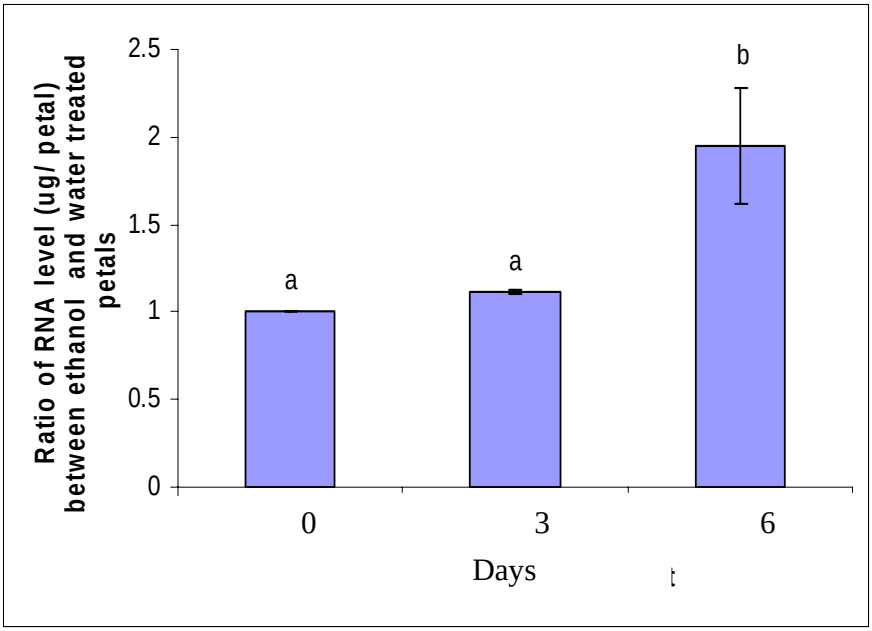


Figure 4. 17 Effect of ethanol (6%) treatment on the total RNA content extracted from isolated petunia petals

Detached petals from open petunia flowers were held in vials of distilled water or 6% ethanol in a growth room. Four or five flowers were used in each treatment. Results are expressed as means ± SE. The values with different letters (a or b) were significantly different using ($p < 0.05$) based on Tukey's method.

4.2.4.2 Optimization of real-time PCR protocol for Cu-Zn SOD and GAPDH genes of petunia

Conditions for real-time PCR were optimized with regard to primer combinations, cDNA concentration and annealing temperatures. Four different combinations of the reverse and forward primers (two by two combinations of F_1, F_2, R_1 and R_2), three annealing temperatures (55, 60 and 65°C) and different cDNA concentrations were originally tested to amplify both gene fragments of Cu-Zn SOD and GAPDH. The optimized conditions should give good amplification efficiency, lower non-specific amplification and a relatively longer amplification fragment.

4.2.4.2.1 Effect of primer combinations on gene amplification

While the primer pair F_1R_2 of Cu-Zn SOD had the slightly higher amplification efficiency, all the four primer combinations for the target gene Cu-Zn SOD generated typical PCR accumulation profiles, with a longer exponential phase and higher plateau value (*Figure 4.18 A*). To determine whether PCR amplification produced multiple amplicons, melting curve analysis was also performed. The melting curves revealed that each of the four primer pairs for Cu-Zn SOD produced only one main amplicon with melting curve temperature ranging from 76˚C to 78˚C (*Figure 4.18 B*). Although a small peak with melting temperature of about 84˚C did appear in each PCR reaction for all the four primer pairs, its intensity was very low in comparison with the main amplicon.

The quantification curves showed that cDNAs synthesized from all the four primer combinations for GAPDH gene also resulted in ideal PCR accumulating profiles (*Figures 4.19 A*). The primer pair F_2R_2 had the highest amplification efficiency, regarding the florescence value in the amplification curve, of main product, but an unexpected melting

curve appeared (*Figures 4.19 A and B*). In PCR reactions for the other three primer pairs, particularly F_2R_1, only a minor peak was observed.

Results of agarose gel analyses showed that there were no primer-dimers and non-specific PCR products using primer pairs F_1R_1 and F_1R_2 for Cu-Zn SOD gene. Also using F_1R_1, F_2R_1 and F_2R_2 for GAPDH gene there were no non-specific products. However, using F_1R_2 for GAPDH gene, F_2R_1 and F_2R_2 for Cu-Zn SOD gene, some non-specific bands or primer-dimers, appeared below the main bands (*Plate 4.18*). These were not observed through melting curve analysis (*Figures 4.18 B* and *4.19 B*). Therefore, primer pairs F_1/R_2 for Cu-Zn SOD gene and F_2/R_1 for GAPDH gene respectively were selected for further real-time PCR analysis to quantify Cu-Zn SOD and GAPDH gene expression.

4.2.4.2.2 Real-time PCR amplification efficiency of target gene (Cu-Zn SOD) and reference genes

In order to quantify DNA from different treatments, total RNA isolated from petunia petals was reverse transcribed to cDNA. Then a serial dilution of 1-fold to 10000-fold of the same first strand cDNA was firstly amplified for Cu-Zn SOD and GAPDH gene fragments using real-time PCR. A standard curve was obtained by plotting the logarithm of different concentrations of cDNAs versus the cross point (cycle number) required to elevate the fluorescence signal above the threshold. The quality of real-time PCR data is assessed by measuring the efficiency of PCR and correlation coefficients of standard curves.

Typical standard curves over five orders of magnitude (from 1-fold to 10000-fold) for the real-time amplification of both Cu-Zn SOD and GAPDH gene fragments using cDNAs of petunia petals were obtained (*Figures 4.20* and *4.22*). Quantification showed a high linear relation since the correlation coefficient R^2 was bigger than 0.99 between the log values of cDNA and real-time PCR threshold cycles over the range of DNA concentration examined (*Figures 4.21* and *4.23*). The real-time PCR efficiency rates were high since

the amplification efficiencies of the real-time PCR reaction, as calculated from the slopes which were -3.509 for Cu-Zn SOD and -3.499 for GAPDH according to the equation [$E = 10^{(-1/slope)}$], reached 92.3% and 93.1% respectively in the range from 1-fold to 10000-fold cDNA input. Two house-keeping genes (18S rRNA and actin) were also used for accurate quantification of the expression of the target gene. Similarly, the high correlation coefficiency of about 99.07% and 99.86%, respectively for the reference genes actin and 18S rRNA, were observed (*Figures 4.25* and *4.27*). The efficiencies of the PCR reactions for actin and 18S RNA genes were 85.5% and 88.4%, which were calculated from the related slopes -2.938 and -3.636, respectively. In most of these analyses, only one PCR product was detected in terms of melting curves and the results from agarose gel electrophoresis (*Figures 4.20 B, 4.22 B, 4.24 B, 4.26 B, 4.21, 4.23, 4.25* and *4.27*).

4.2.4.2.3 Determination of appropriate cDNA concentration for gene amplification

Using diluted cDNA as real-time PCR template is considered an efficient way to eliminate the inhibitory effect of some reagents used in cDNA synthesis. The representative progress curves for real-time amplification of Cu-Zn SOD and GAPDH gene fragments from known amounts (1-, 10-, 100-, 1000- and 10000-fold) of cDNA from petunia petals showed that no remarkable inhibitory effect was observed using either undiluted cDNA or 10- to 10000-fold diluted cDNAs as template, all with normal amplification curves (*Figures 4.20* and *21*). Result of melting curve analysis showed that reactions using undiluted and 10-fold diluted cDNA templates for Cu-Zn SOD gene generated a small peak of non-specific PCR product. Its melting temperature was at around 85^{o}C. Those using 100- to 10000-fold diluted cDNAs generated only one specific product. However, the increased cDNA dilution factor greatly increased the threshold cycle (C_t) as well, making the precise quantification of gene expression difficult. This is a problem particularly during flower senescence when a decreased level of general gene expression is expected to occur.

Therefore, a more detailed series of cDNA dilution between 10-fold and 100-fold was tested. 50-fold dilution was also satisfactory, with similar amplification curves to that of the 100-fold cDNA dilution (*Figures 4.28* and *4.29*). The specificity of these PCR reactions was also confirmed using agarose gel eletrophoresis. The negative control without template did not produce any amplicons for both Cu-Zn SOD and GAPDH genes as shown on the 2% agarose gels after electrophoresis (*Plate 4.19*). Therefore, 50- to 100-fold diluted cDNA was thought to be ideal for quantifying Cu-Zn SOD gene expression in petunia petals using real-time PCR assays.

4.2.4.2.4 Effect of annealing temperature on real-time PCR amplification

Only one expected product from three annealing temperatures was revealed using the melting curve (*Figures 4.30, 4.31* and *4.32*) and agarose gel electrophoresis analyses for both the target gene (Cu-Zn SOD) and reference gene (GAPDH) (*Plate 4.20*). However, 55 ˚C and 60˚C were the optimal annealing temperatures for Cu-Zn SOD and GAPDH genes considering their Ct values and the amplification curves. Agarose gel analysis also supported this in terms of the brightness of the bands. In comparison, 65˚C was too high for amplification of both genes since the amplification efficiency and intensity of the PCR products decreased.

In summary, 50-fold cDNA dilution, 55˚C / 60˚C annealing temperature and primer pairs of F_1R_2 for Cu-Zn SOD gene and F_2R_1 for GAPDH gene were selected and used for further real-time PCR experiments in this research.

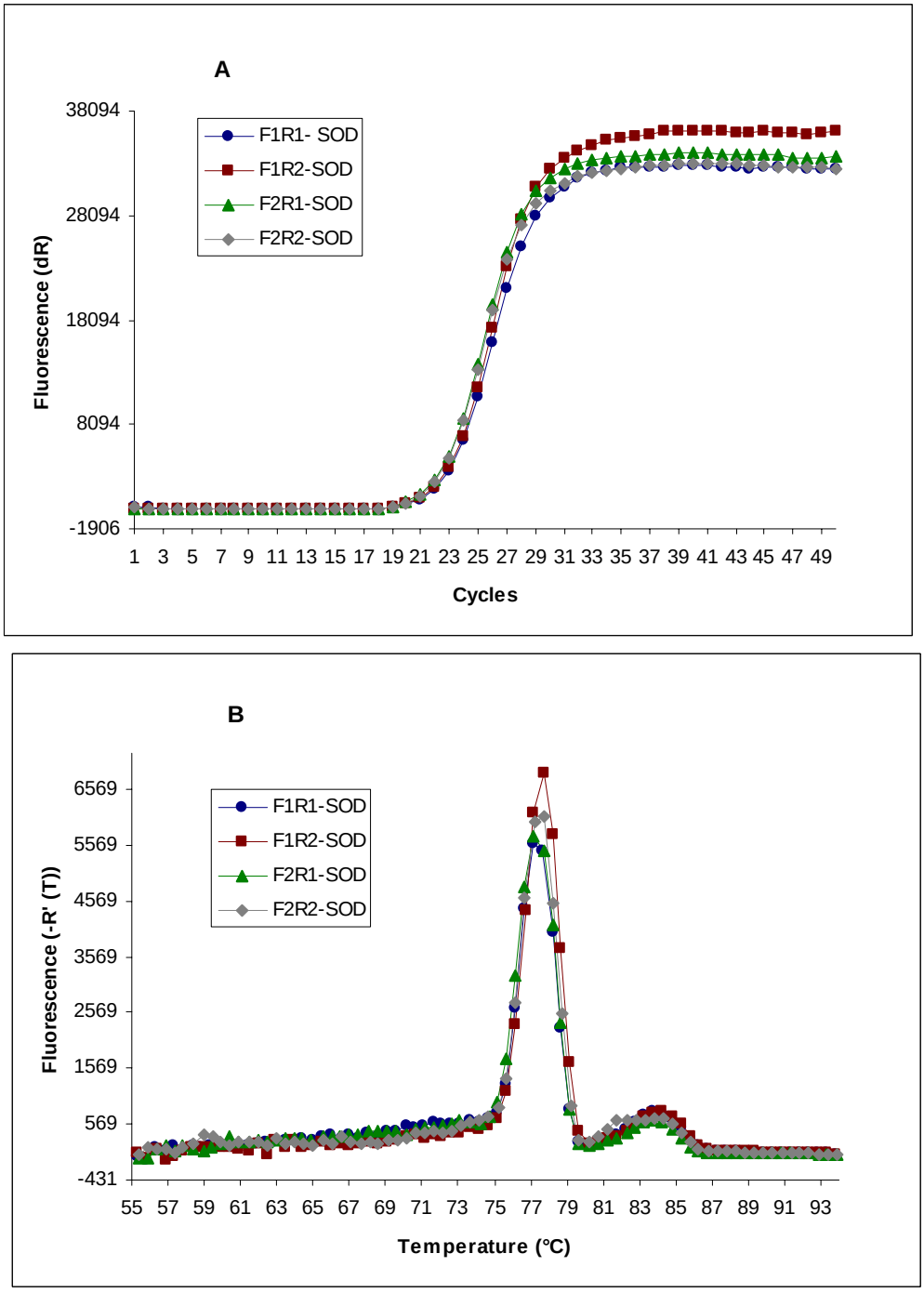


Figure 4. 18 Real-time RT-PCR amplification of a petunia Cu-Zn SOD gene fragment using different combinations of reverse (R) primers with forward (F) primers.

Upper panel (A): quantification curves; lower panel (B): melting curves.

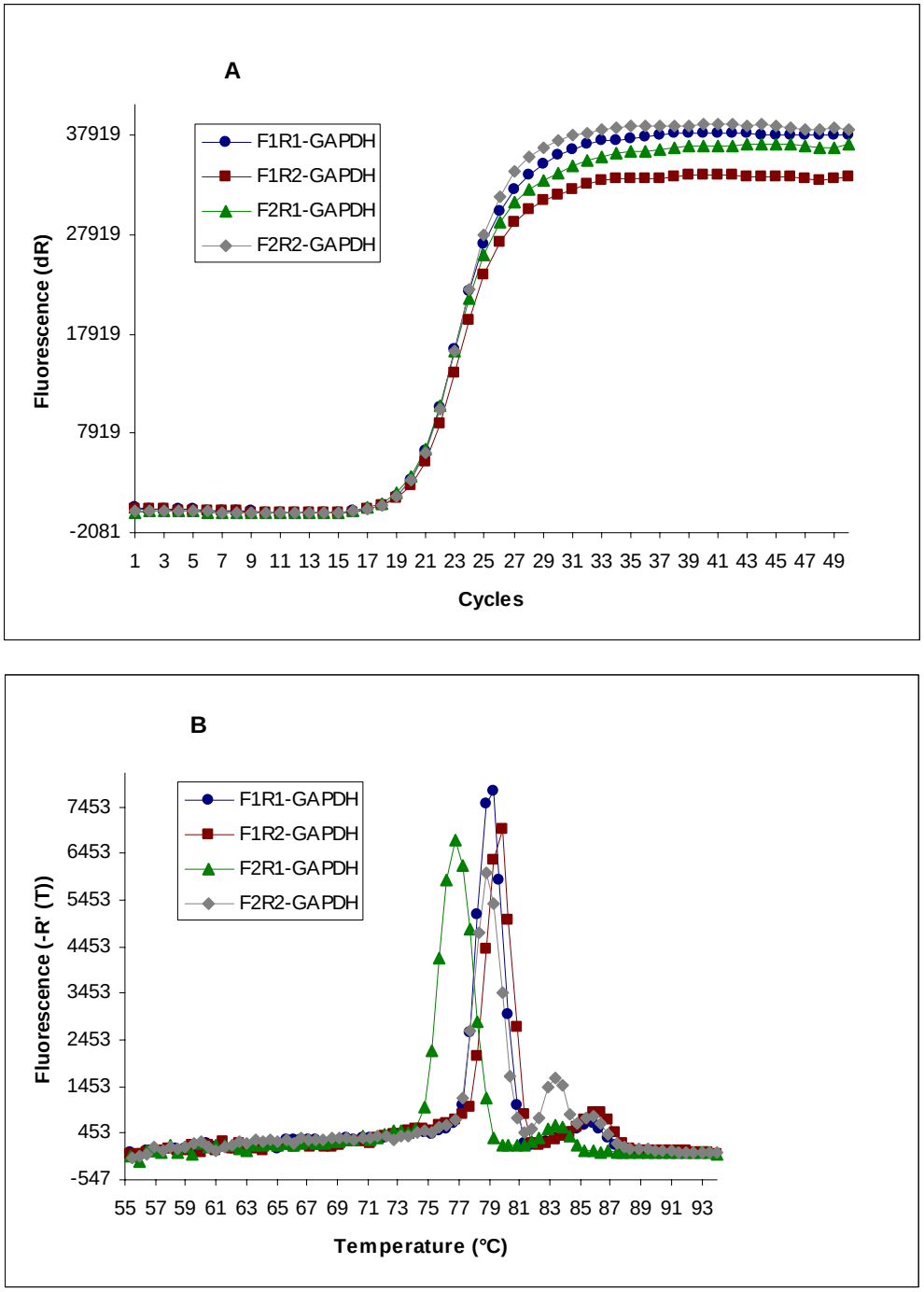


Figure 4. 19 Real-time RT-PCR amplification of a GAPDH gene fragment using different combinations of reverse (R) primers and forward (F) primers

Upper panel (A): quantification curves; lower panel (B): melting curves.

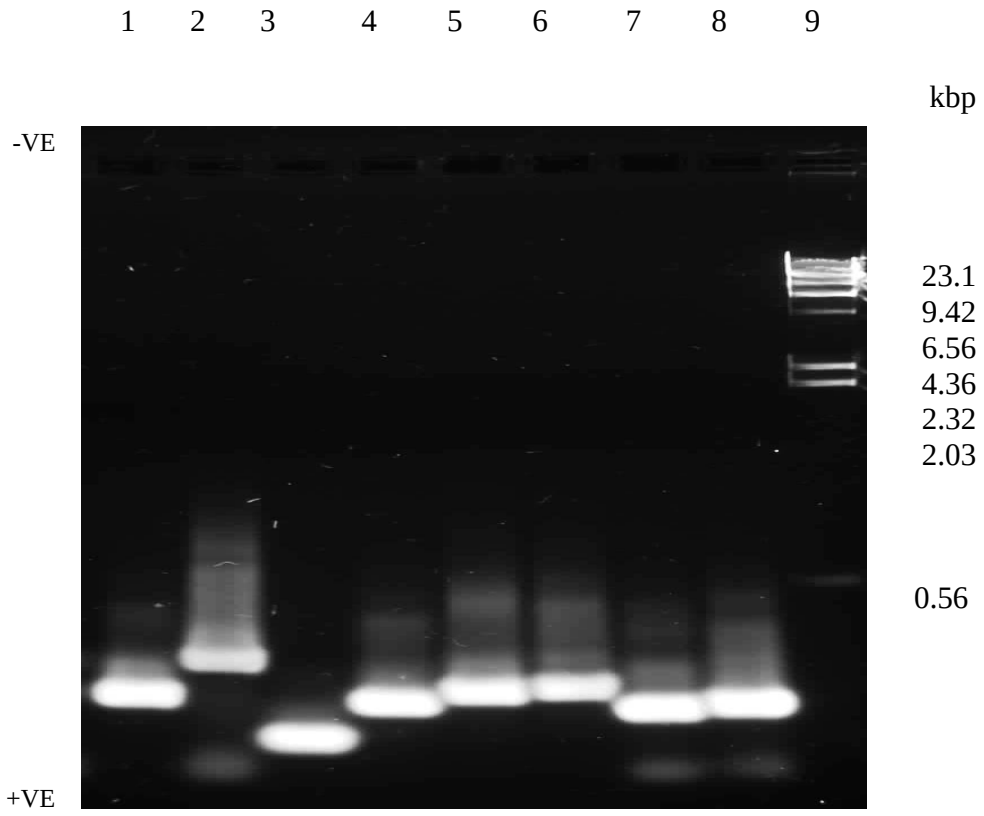


Plate 4. 18 Agarose gel electrophoresis after real-time PCR amplification of petunia petal Cu-Zn SOD and GAPDH gene fragments

Five µl of real-time PCR products were subjected to agarose (1%) gel electrophoresis at 80 V for 1 hour. Lanes 1-4: different primer pairs for GAPDH gene (F_1R_1; F_1R_2; F_2R_1; F_2R_2); Lanes 5-8: different primer pairs for Cu-Zn SOD gene (F_1R_1; F_1R_2; F_2R_1; F_2R_2); Lane 9: DNA molecular weight standards (5 µl loaded).

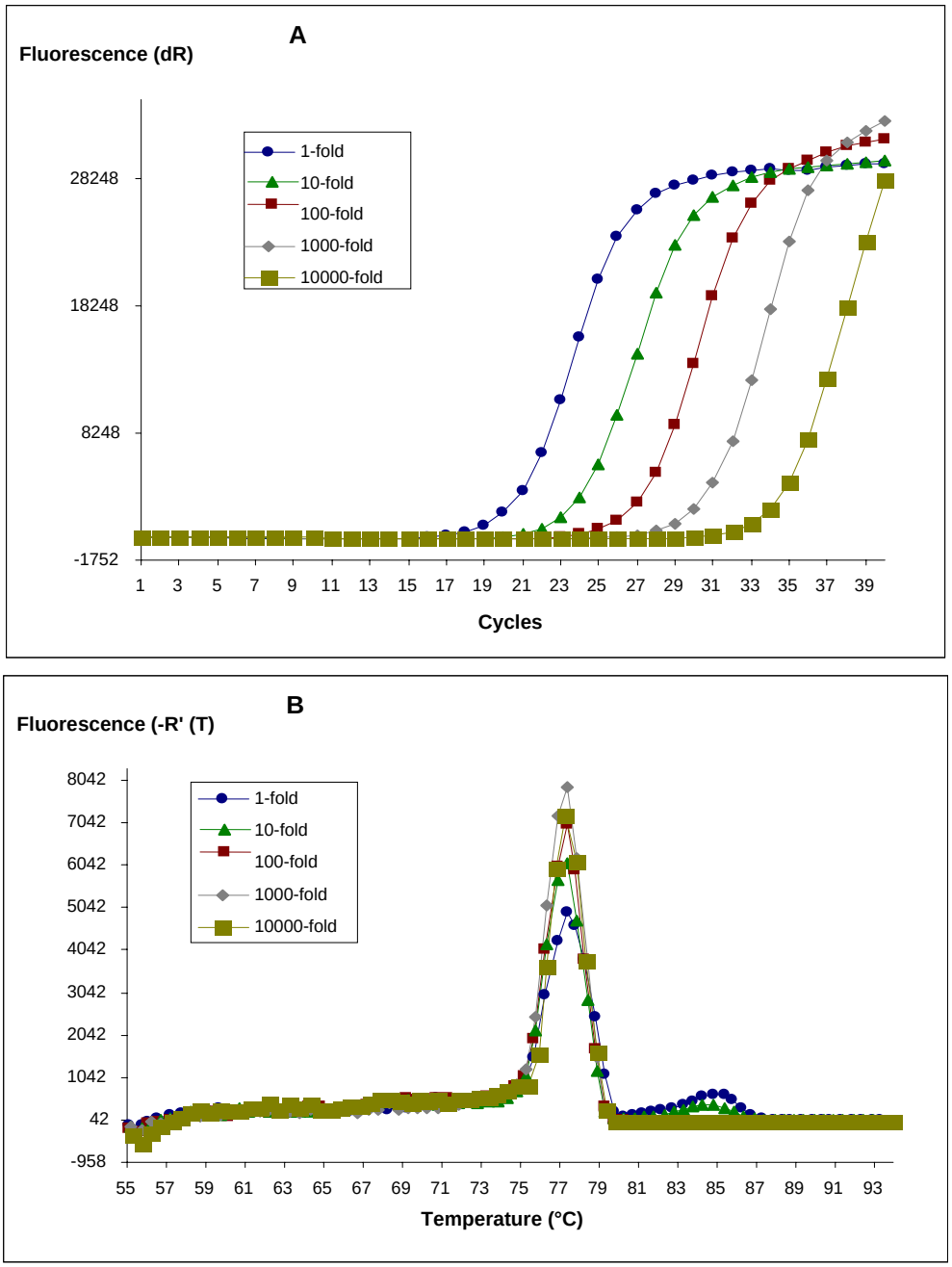
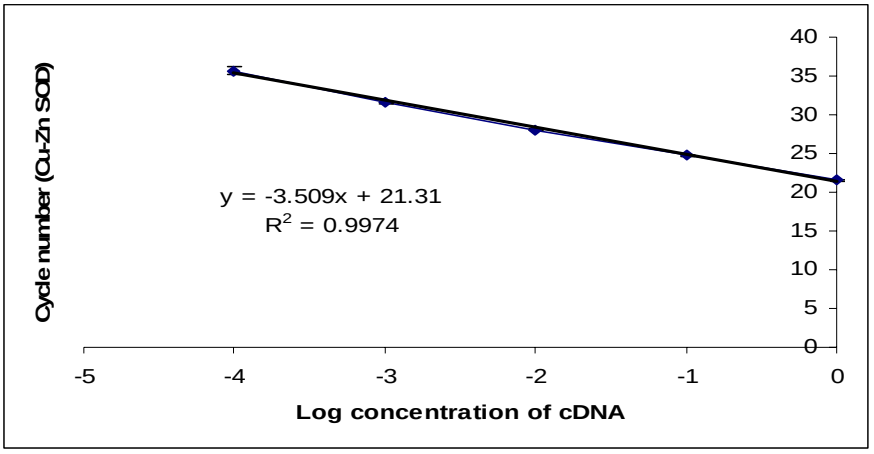


Figure 4. 20 Real-time PCR amplification of a petunia Cu-Zn SOD gene fragment using serially diluted petunia petal cDNA templates

Upper panel (A): amplification curves; lower panel (B): melting curves



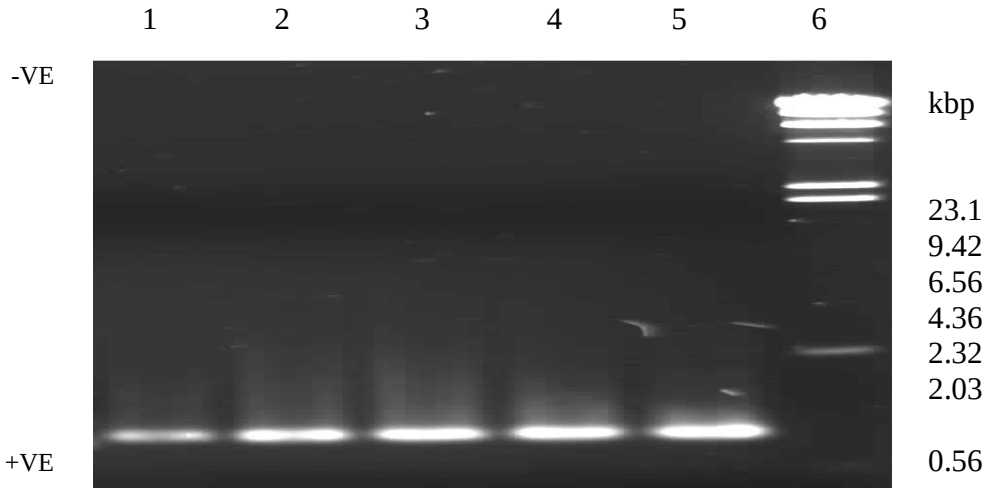


Figure 4. 21 Standard curve of real-time PCR amplification of a Cu-Zn SOD gene fragment (upper figure) and corresponding agarose gel after electrophoresis (lower figure)

Lanes 1-5: different concentrations of petunia petal cDNA templates from 10000-, 1000-, 100-, 10- and 1-fold dilution; Lane 6: DNA molecular weight standards. The results in the standard curve are expressed as means ± SE. for three determinations. Ct cycles versus cDNA (from reverse transcribed total RNA) concentration input were plotted to calculate the slope. The corresponding real-time PCR efficiencies were calculated according to the equation: $E = 10^{(-1/slope)}$. There is a linear relationship between Ct values and log concentrations of cDNA. R^2 value and slope are 99.74% and -3.509 respectively.

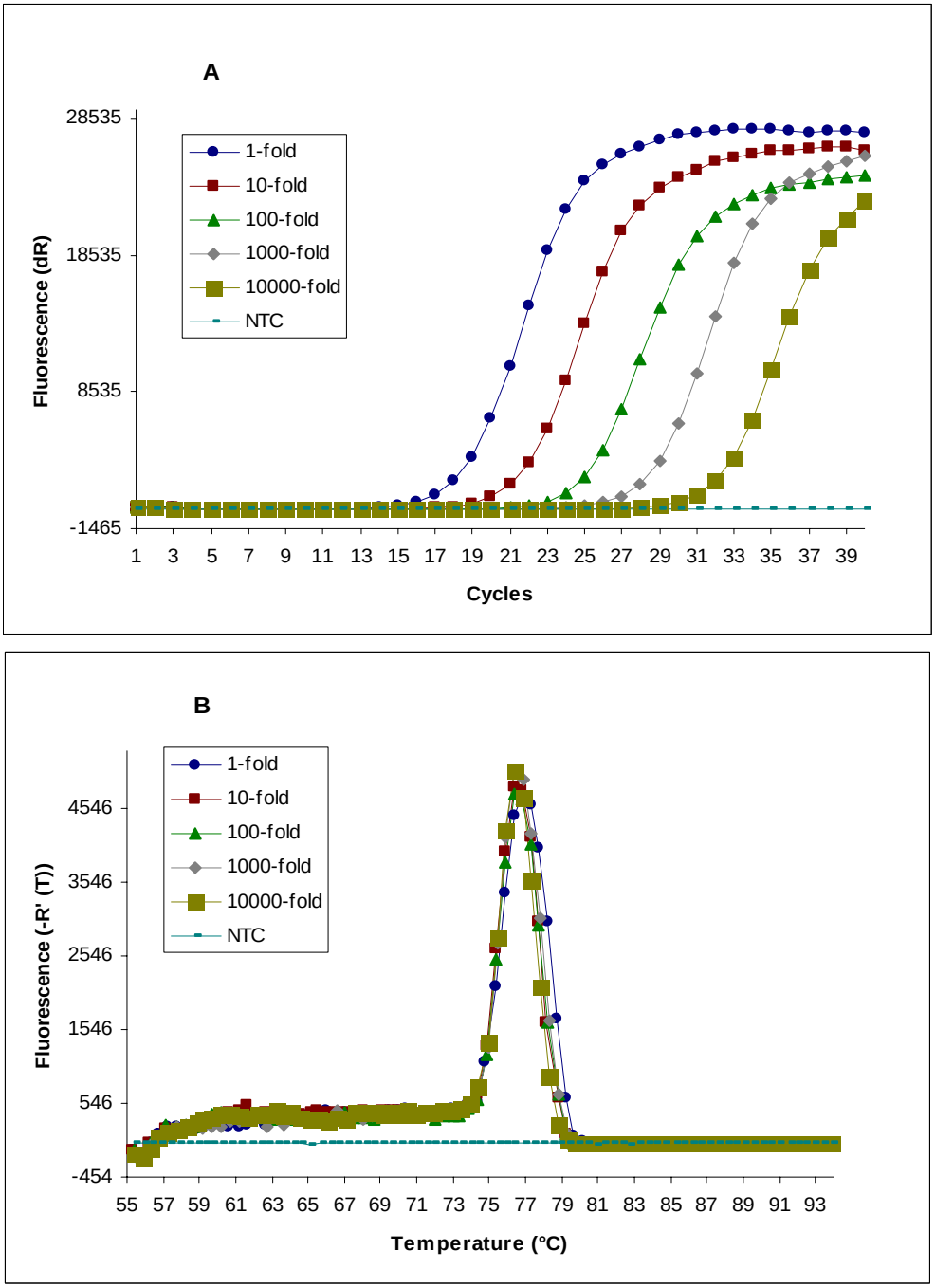
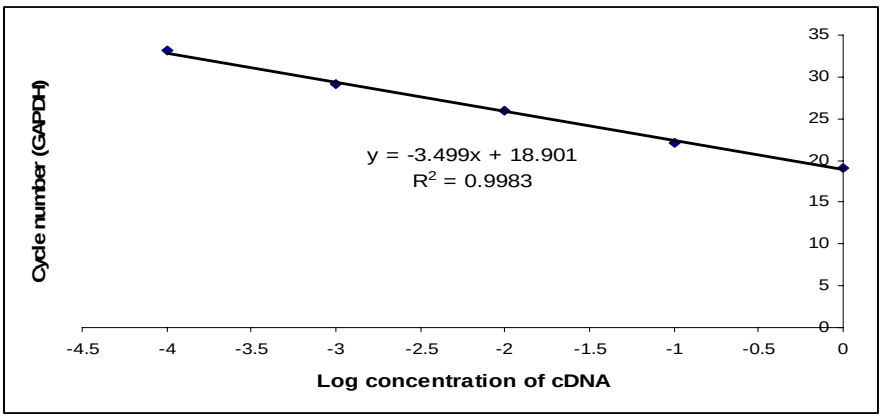


Figure 4. 22 Effect of serially diluted petunia petal cDNA on real-time PCR amplification of a GAPDH gene fragment

Upper panel (A): amplification curves; lower panel (B): melting curves; NTC: no template control.



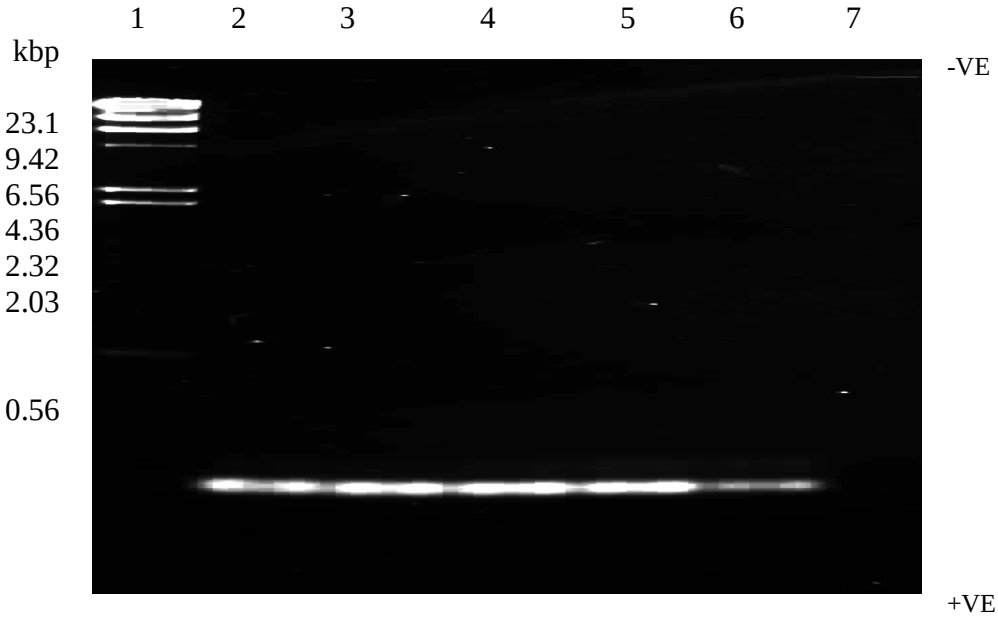


Figure 4. 23 Standard curve of real-time PCR amplification of a GAPDH gene fragment (upper figure) and corresponding agarose gel after electrophoresis (lower figure)

Lane 1: DNA molecular weight standards; Lanes 2-6: different concentrations of petunia petals cDNA templates from 1-, 10-, 100-, 1000- and 10000-fold dilution; Lane 7: control (no cDNA template). The results are expressed as means ± SE. Ct cycles versus cDNA (from reverse transcribed total RNA) concentration input were plotted to calculate the slope. The corresponding real-time PCR efficiencies were calculated according to the equation: $E = 10^{(-1/slope)}$. There is a linear relationship between Ct values and log concentrations of cDNA. R^2 value and slope are 99.83% and -3.499, respectively.

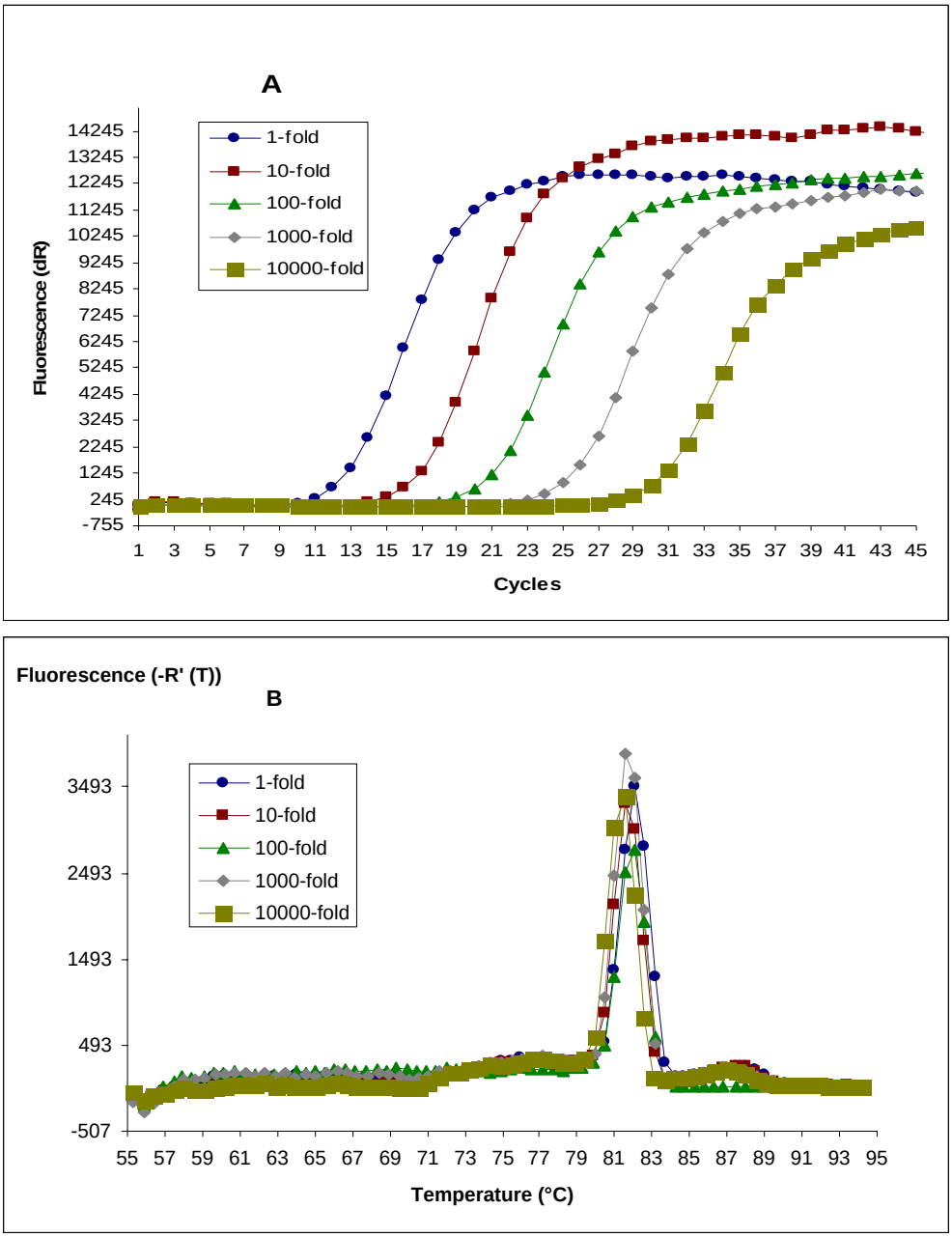


Figure 4. 24 Effect of serially diluted petunia petal cDNA templates on real-time PCR amplification of an 18S rRNA gene fragment

Upper panel (A): amplification curves; lower panel (B): melting curves

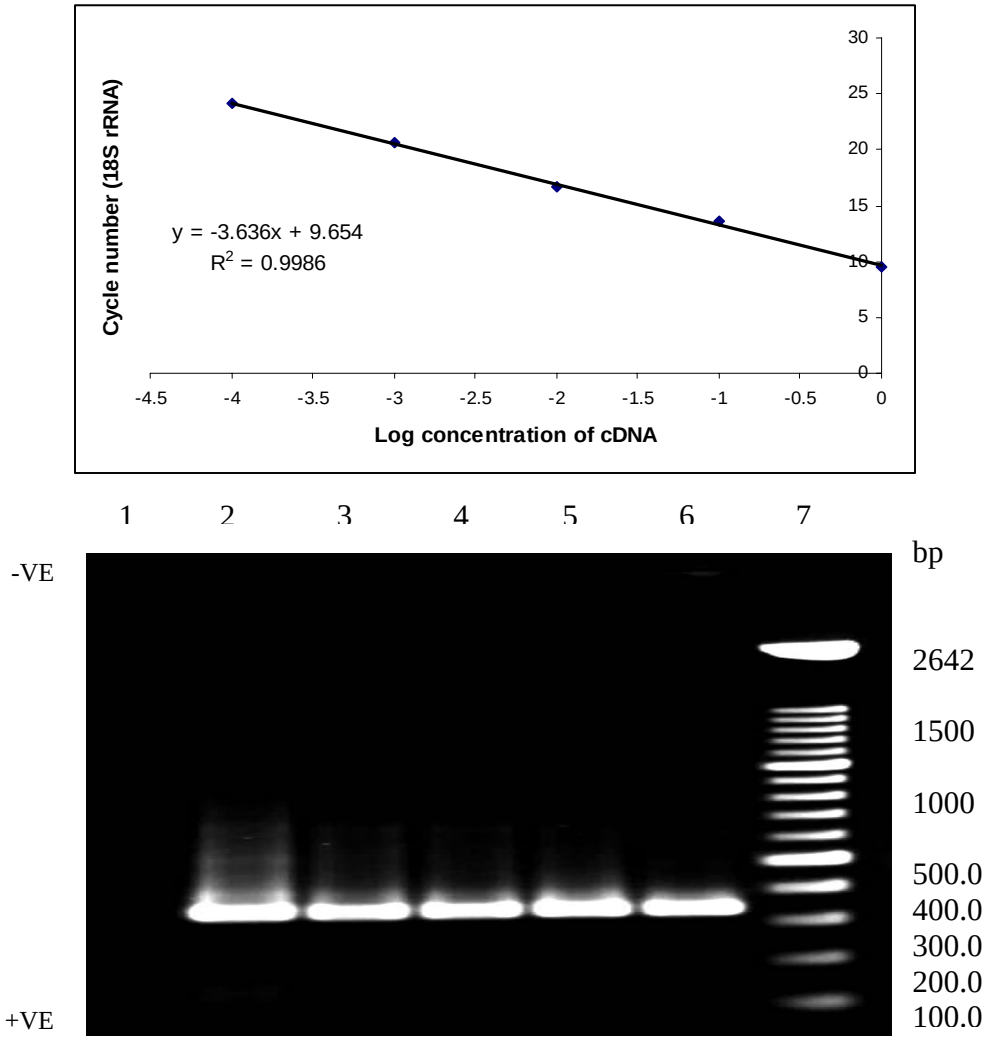
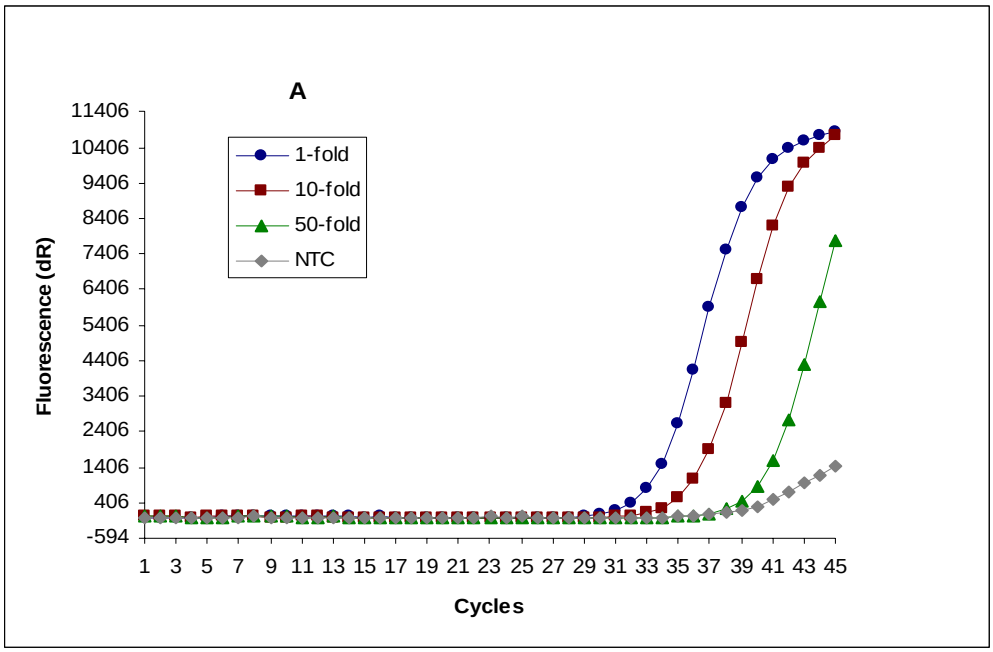


Figure 4. 25 Standard curve of real-time PCR amplification of an 18S rRNA gene fragment (upper figure) and corresponding agarose gel after electrophoresis (lower figure)

Lane 1: control (no cDNA template); Lanes 2-6: different petunia petal cDNA templates from 1-, 10-, 100-, 1000- and 10000-fold dilution; Lane 7: DNA markers. The results are expressed as means ± SE. Ct cycles versus cDNA (from reverse transcribed total RNA) concentration input were plotted to calculate the slope. The corresponding real-time PCR efficiencies were calculated according to the equation: $E = 10^{(-1/slope)}$. There is a linear relationship between Ct values and log concentrations of cDNA. R^2 value and slope are 99.86% and -3.636, respectively.



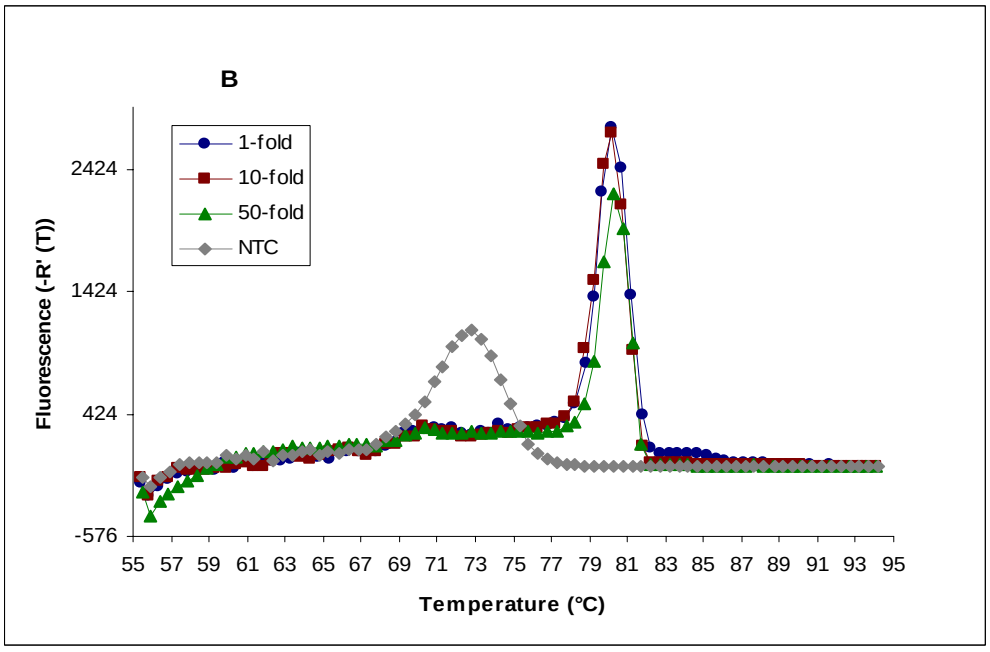


Figure 4. 26 Effects of serially diluted petunia petal cDNA templates on real-time PCR amplification of an actin gene fragment

Upper panel (A): amplification curves; lower panel (B): melting curves; NTC: no template control.

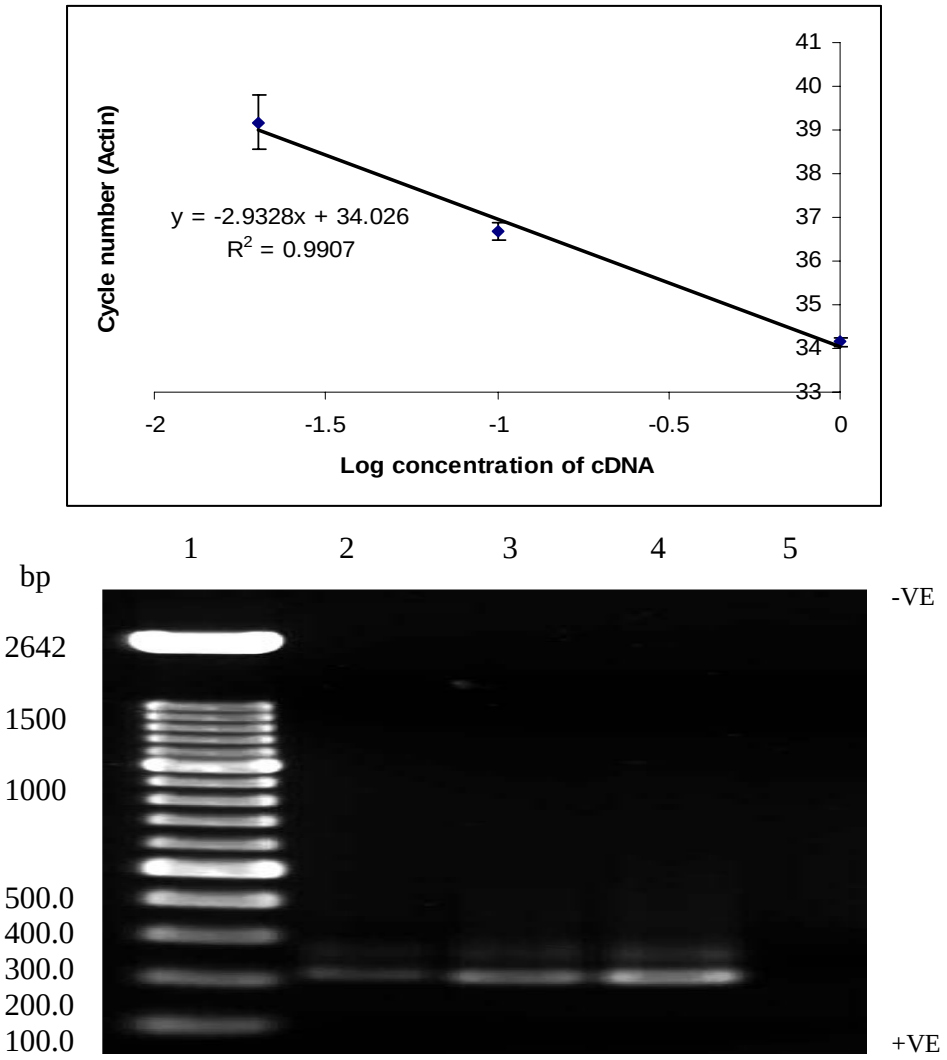


Figure 4. 27 Standard curve of real-time PCR amplification of an actin gene fragment (upper figure) and corresponding agarose gel after electrophoresis (lower figure)

Lane 1: DNA markers; Lanes 2-4: different concentrations of petunia petal cDNA templates from 50-, 10- and 1-fold dilution; Lane 5: control (no cDNA template). The results are expressed as means ± SE. for three determinations. Ct cycles versus cDNA (from reverse transcribed total RNA) concentration input were plotted to calculate the slope. The corresponding real-time PCR efficiencies were calculated according to the equation: $E = 10^{(-1/slope)}$. There is a linear relationship between Ct values and log concentrations of cDNA with R^2 value and slope are 99.07% and -2.9328, respectively.

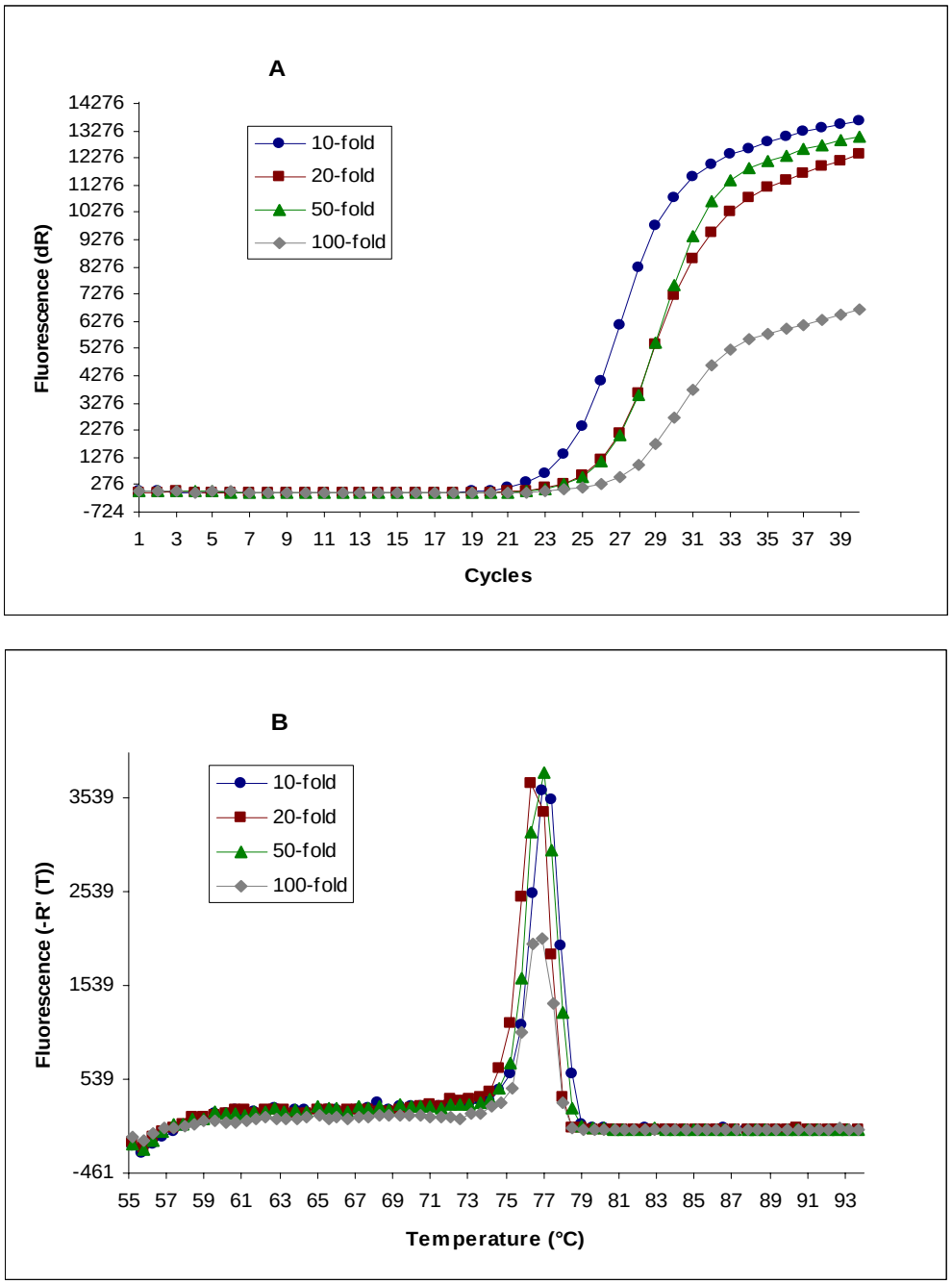


Figure 4. 28 Optimization of RT-PCR of a Cu-Zn SOD gene fragment with regard to different concentrations of cDNA

Upper panel (A): quantification curves; lower panel (B): melting curves.

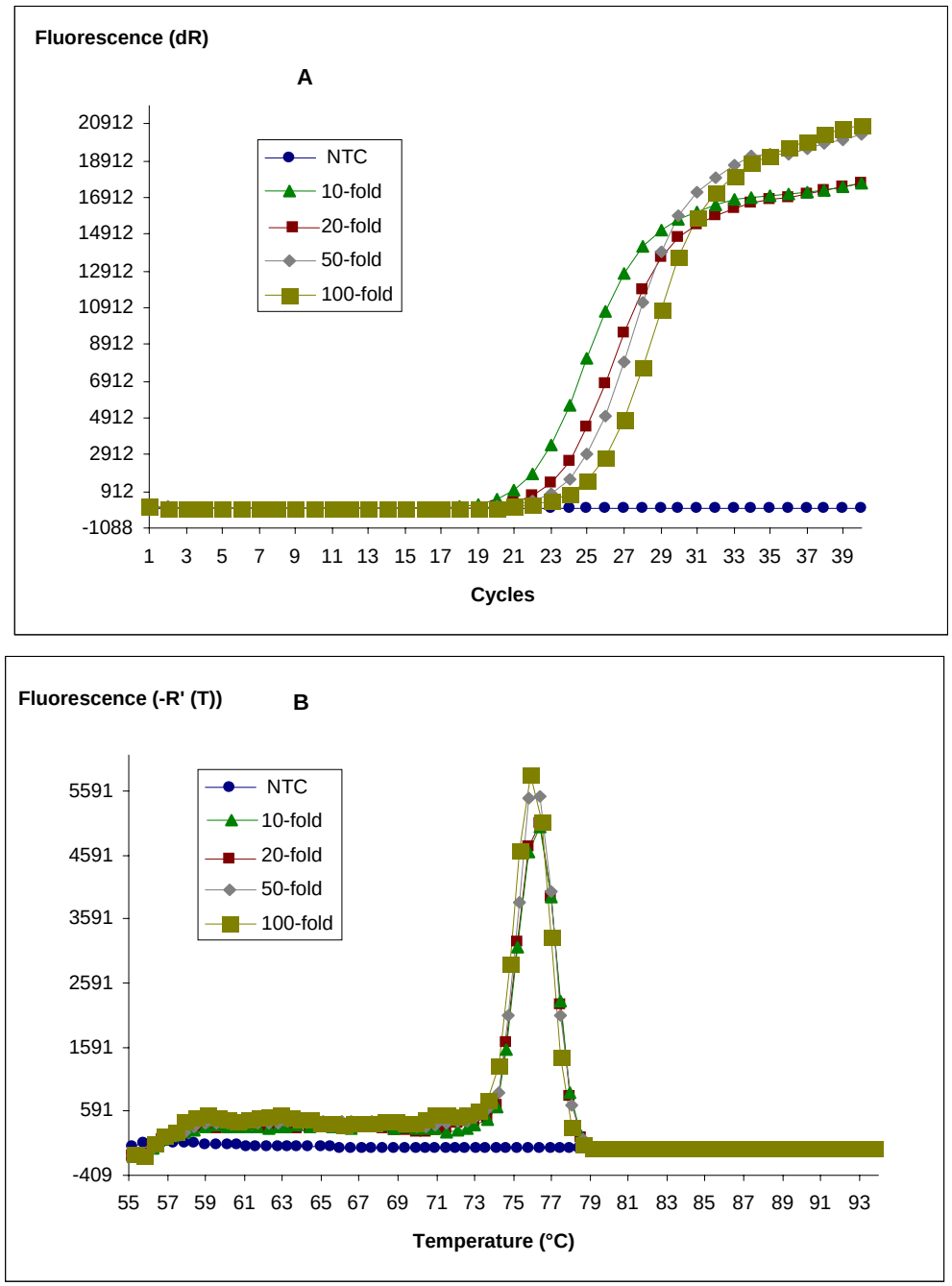


Figure 4. 29 Optimization of RT-PCR of a GAPDH gene fragment with regard to different concentrations of cDNA

Upper panel (A): quantification curves; lower panel (B): melting curves; NTC: no template control.

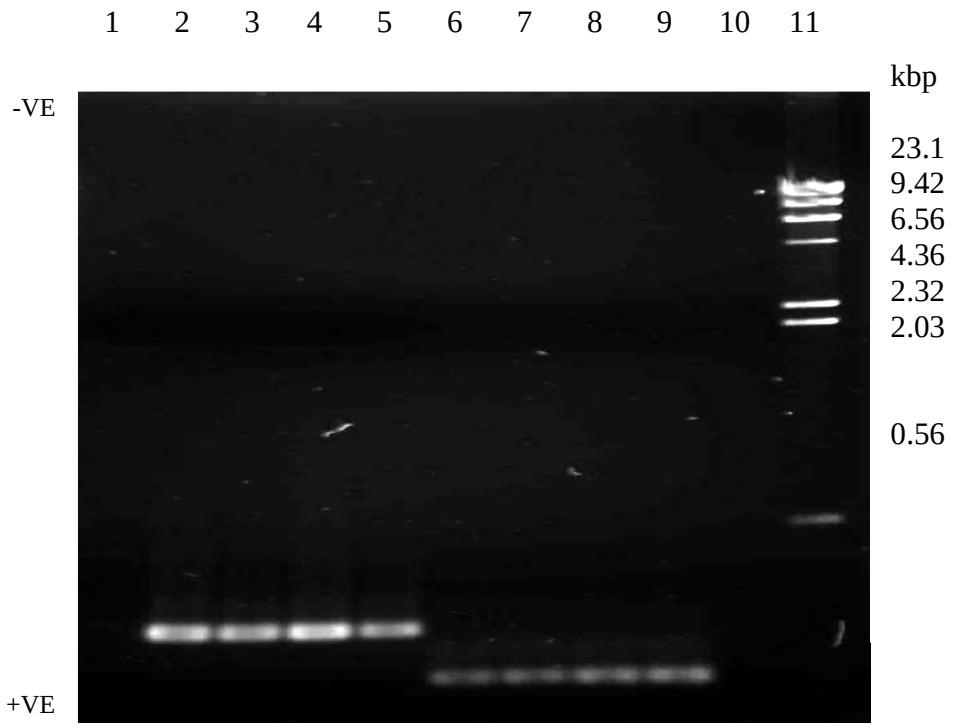


Plate 4. 19 Agarose gel electrophoresis of real-time PCR products (Cu-Zn SOD and GAPDH gene fragments) using different concentrations of petunia petal cDNA as templates

Lane 1: negative control for Cu-Zn SOD gene (absence of template); Lanes 2-5: 10-, 20-, 50- and 100-fold diluted cDNA of petunia Cu-Zn SOD gene; Lanes 6-9: 100-, 50-, 20- and 10-fold diluted cDNA of petunia GAPDH gene; Lane 10: negative control for GAPDH gene (absence of template); Lane 11: DNA markers.

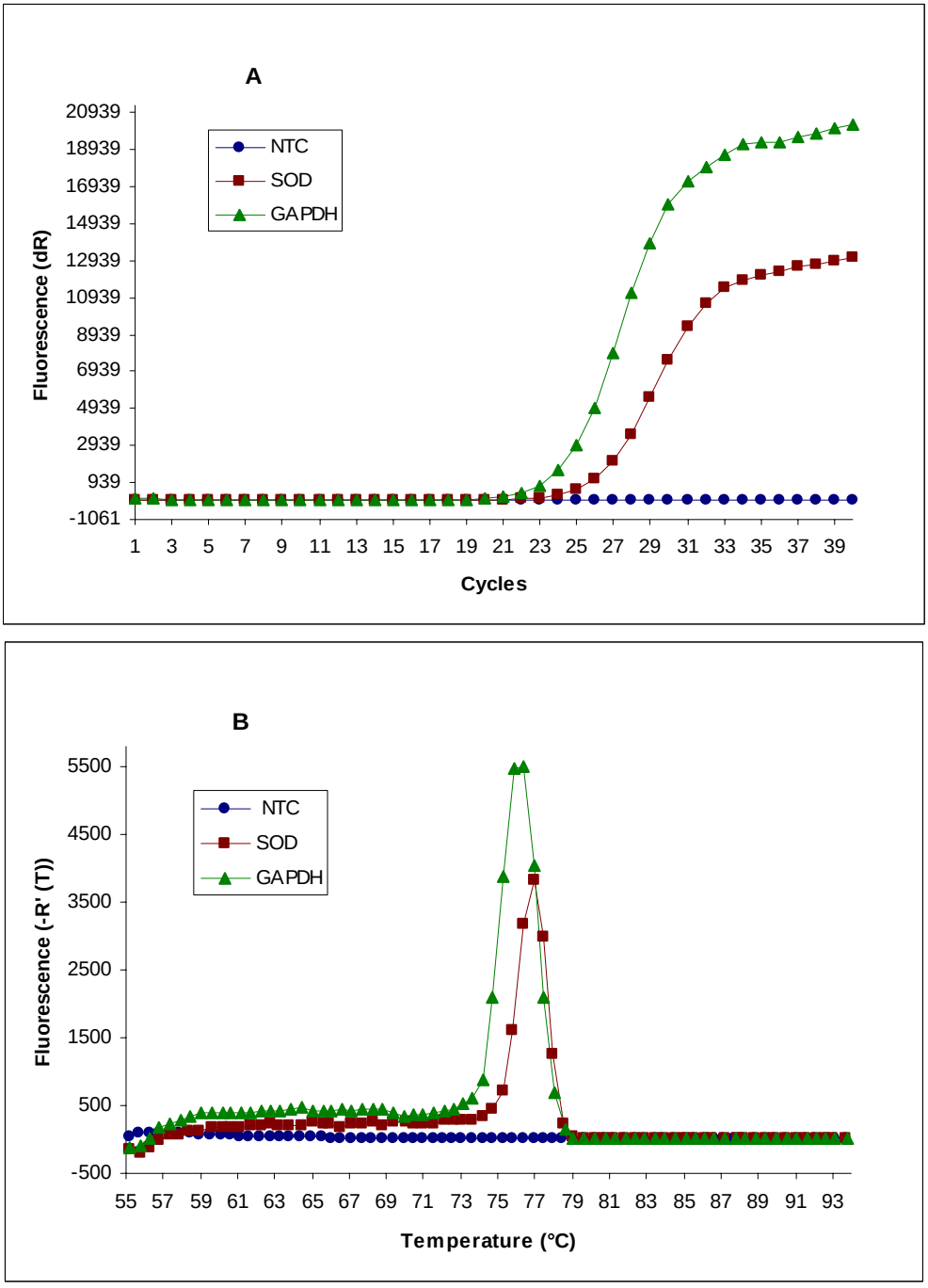


Figure 4. 30 Real-time PCR amplification of petunia Cu-Zn SOD and GAPDH gene fragments at 55˚C as the annealing temperature

The 50-fold diluted petunia petal cDNA as template. NTC: no template control; upper panel (A): quantification curves; lower panel (B): melting curves.

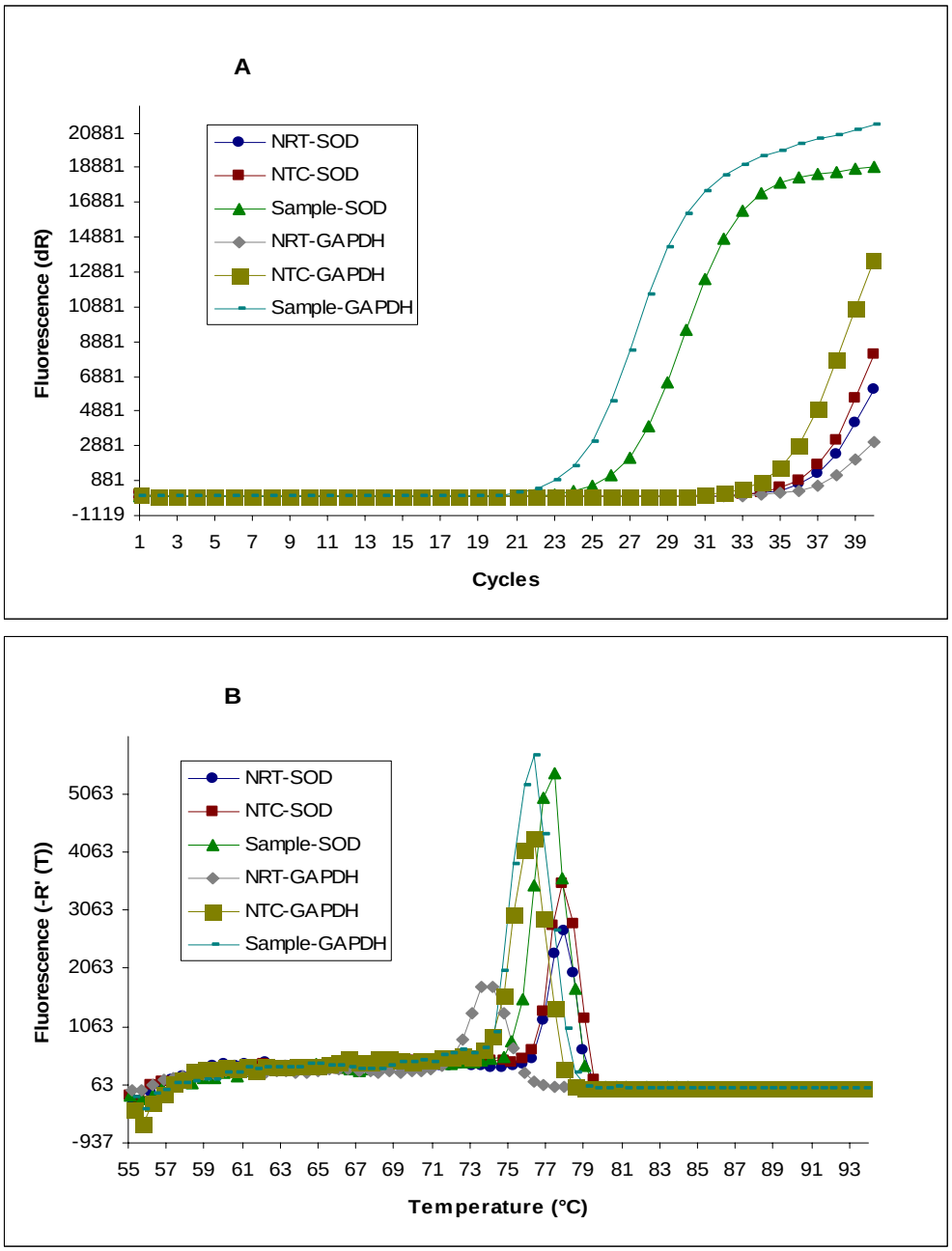
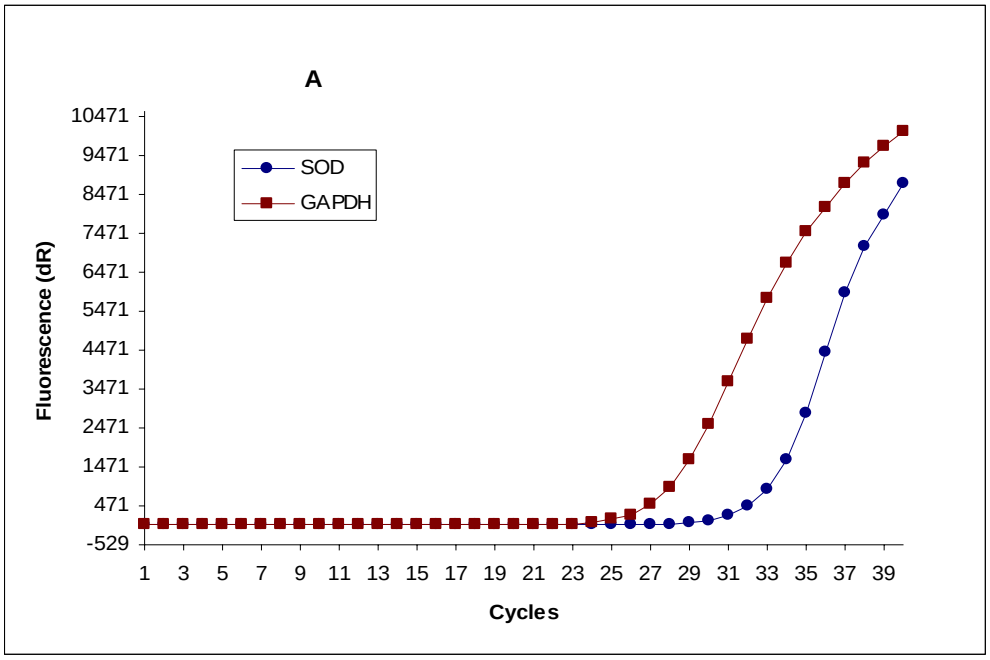


Figure 4. 31 Real-time PCR amplification of petunia Cu-Zn SOD and GAPDH gene fragments at 60˚C as the annealing temperature

The 50-fold diluted petunia petal cDNAs as template. NTC: no template control; NRT: no reverse transcriptase control; upper panel (A): quantification curves; lower panel (B): melting curves.



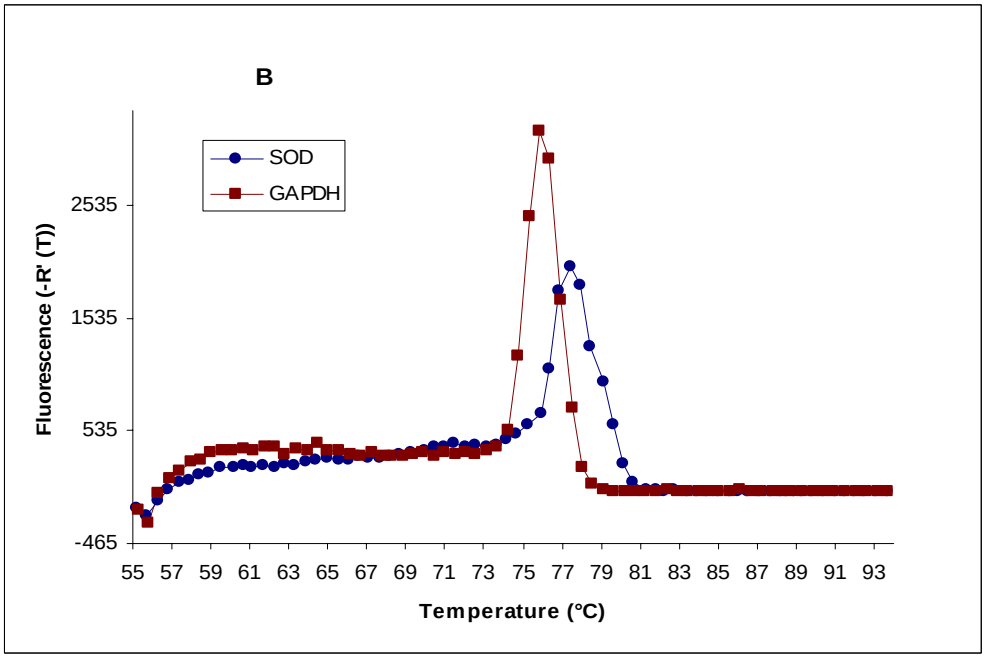


Figure 4. 32 Real-time PCR amplification of petunia Cu-Zn SOD and GAPDH gene fragments at 65˚C as the annealing temperature

The 50-fold diluted petunia petal cDNA as template. Upper panel (A): quantification curves; lower panel (B): melting curves.

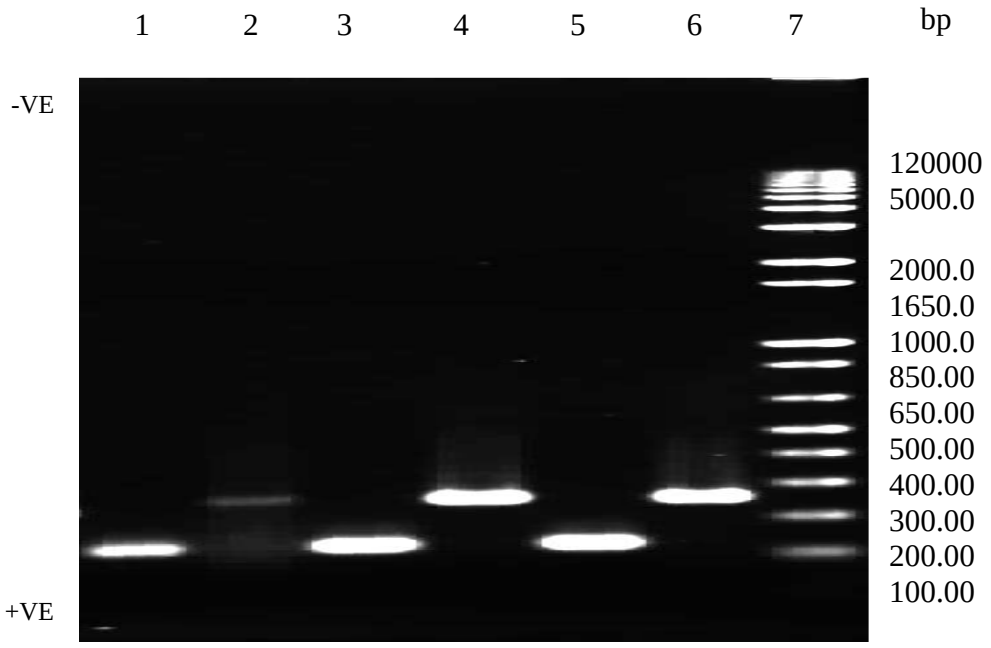


Plate 4. 20 Agarose gel electrophoresis after real-time PCR amplification of petunia Cu-Zn SOD and GAPDH gene fragments at different annealing temperatures

Real-time PCR products of Cu-Zn SOD and GAPDH gene fragments were subjected to agarose (2%) gel electrophoresis (80 V for 1.5 hours).

Lanes 1-2: GAPDH and Cu-Zn SOD gene fragments respectively amplified at 65˚C; Lanes 3-4: GAPDH and Cu-Zn SOD gene fragments respectively amplified at 60˚C; Lanes 5-6: GAPDH and Cu-Zn SOD gene fragments respectively amplified at 55˚C; Lane 7: 100 kb DNA markers (3 µl loaded).

4.2.4.3 Isolation and sequence analysis of a putative Cu-Zn SOD gene fragment

A single PCR product corresponding to a Cu-Zn SOD gene fragment was obtained using petunia petal cDNA as the PCR template. Eight sequences were obtained from purified PCR products of four different petal samples using both forward and reverse primers. After multiple sequence alignments as shown in *Figure 4.33*, a consensus sequence of 198 bp for a putative Cu-Zn SOD gene fragment was generated as follows:

TTCACCACCACAAGCACTATCAATTCTTTCTTATTCCCAATTGCTTCCTCTAAC
ACCAACTCTAGCCCTTCACTTTCCTCTTCTTTCCATGGTGTTTCCCTTAAAGTC
AAGTCAAAAACTCCYCAATCTTTGACCCTTTCATCTGTCACTTCTYCTAAAAC
TTTCATTGTTTTTGCTGCTACTAAGAAAGCTGTTGCT

The predicted amino acid sequence of this fragment of Cu-Zn SOD in petunia 'hurrah' is:
FTTTST**I**NSFLFPIASSNTNS**S**PSLSSSFHGVSLKVKSKTPQSLTLSSVTSPK**T**FIVFA
ATKKAVA

BLAST search against both the GenBank nucleotide and protein database showed that the putative Cu-Zn SOD sequence obtained in this study was most similar to the chloroplast superoxide dismutase in petunia x hybrida (X14352). It shared about 96% similarity at the nucleotide level along the overlapping sequence region (which is underlined) with the reported Cu-Zn SOD gene sequence in petunia x hybrida.

TCCTTCACATTATCATTCATCACTCACACTATCATCTTAGCCACCTGTGTCTGT
GTGGCATCCATGGCAGCCCATACAATC<u>TTCACCACCACAAGCACTACCAATT
CTTTCTTATTCCCAATTGCTTCCTCTAACACCAACTCTGCCCCTTCACTTTCCT
CTTCTTTCCATGGTGTTTCCCTTAAAGTCAAGTCAAAAACTCCTCAATCATTG
ACCCTTTCATVTGTCACTTCTCCTAAACCTTTCATTCTTTTTGCTGCTACTAAT</u>

AAAGCTGTTGCTGTACTTAAAGGCACTTCCAATGTTGAGGGTGTTGTCACTCT
TACTCAAGATGATGATGGTCCAACCACTGTGAAAGTTCGTATAACTGGACTT
GCTCCTGGACTTCATGGGTTTCATTTGCACGAGTTTGGTGACACTACCAATGG
GTGTATGTCCACAGGACCCCATTTCAATCCTAACGGCTTGACACATGGAGCTC
CTGGAGATGAAGTCCGTCATGCGGGTGACCTGGGAAACATAGAGGCCAATGC
CAGTGGTGTGGCTGAAGCAACACTTGTAGATAATCAGATACCATTGTCTGGT
CCAAACTCAGTCGTTGGAAGAGCGCTTGTAGTTCACGAGCTTGAGGATGATC
TTGGAAAGGGTGGCCACGAACTCAGCCTTACCACTGGAAATGCTGGTGGACG
ATTGGCCTGTGGTGTGGTTGGTTTGACTCCAATATGAAGTCAAGATAGCAGTA
ATTCTTTGGTATGAATTATAATGAGTGGTTATCTCCTCCCCACGTTGCCAATA
AAGCTCTACGTGAATGTATTTTGAGAGATTCTTGATGGACAAATTTGAGCTTG
CATGTACACTTGCCAAGTGTAGTGCACCACTTTTTAACTCAACACTGTAATTG
GAGTCGGCTATTTTACCATCCTTAAGAGTTTTTTCTATC

The predicted amino acid sequence shared 92% similarity with the reported Cu-Zn SOD gene sequence in petunia x hybrida. The overlapping sequence region is underlined (see below). Within the overlapping sequence region (66 amino acids), three amino acid differences were found between them, with I, S and T in petunia hurrah replaced by T, A and P in the reported petunia x hybrida sequence.

MAAHTIFTTTSTTNSFLFPIASSNTNSAPSLSSSFHGVSLKVKSKTPQSLTLSSVTSP
KPFIVFAATKKAVAVLKGTSNVEGVVTLTQDDDGPTTVKVRITGLAPGLHGFHL
HEFGDTTNGCMSTGPHFNPNGLTHGAPGDEVRHAGDLGNIEANASGVAEATLV
DNQIPLSGPNSVVGRALVVHELEDDLGKGGHELSLTTGNAGGRLACGVVGLTPI
(P10792).

The putative Cu-Zn SOD gene fragment obtained in this study showed a very high sequence similarity to the reported Cu-Zn SOD sequence in the petunia hybrid and Cu-Zn SOD homologues in many other plant species. This confirms that the sequence data of this fragment can be used for later quantification of Cu-Zn SOD gene expression in petunia petals.

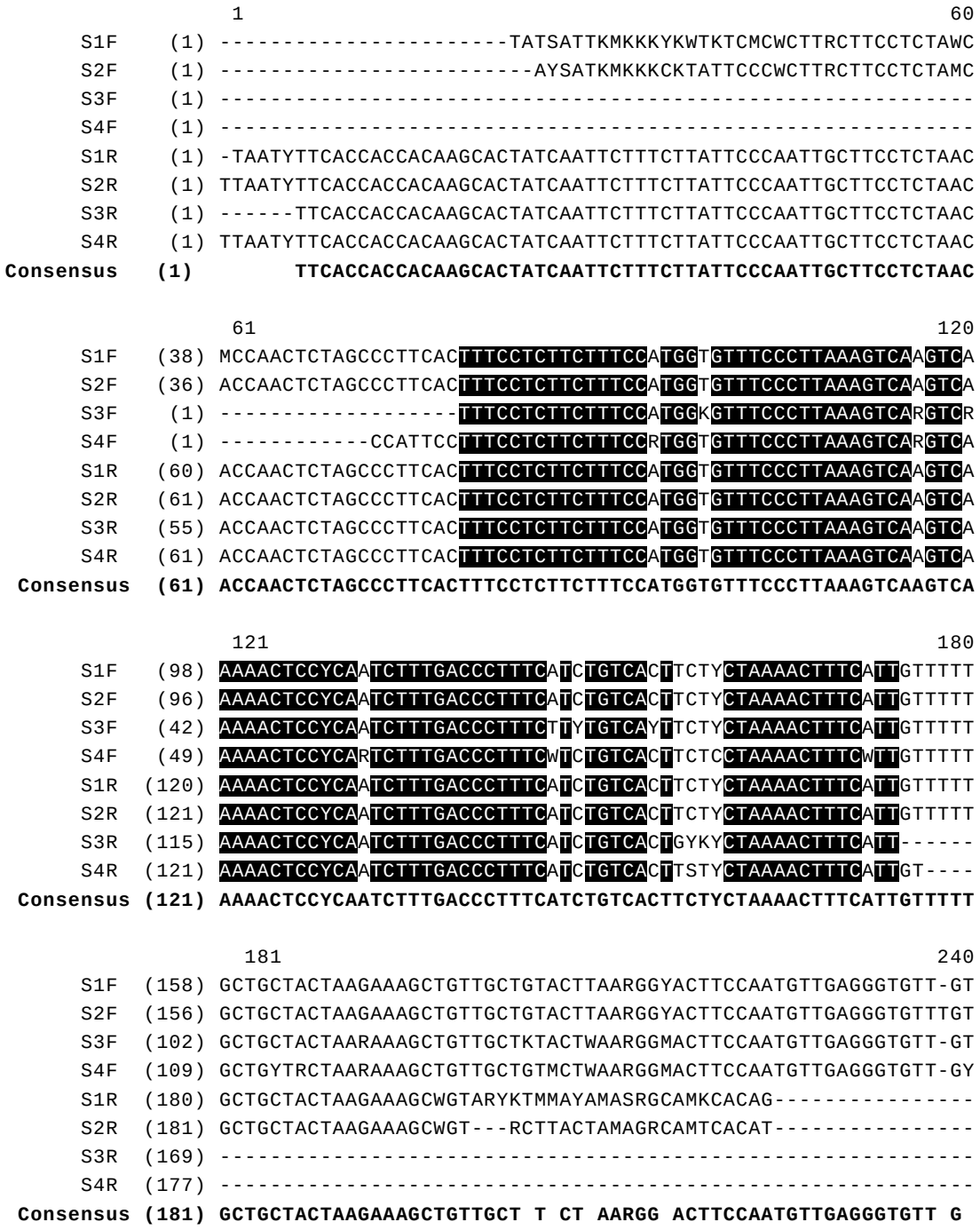


Figure 4. 33 Multiple alignments of putative petunia Cu-Zn SOD sequencing data

S1, S2, S3 and S4: four template samples for sequencing; F and R: forward and reverse primers. Highly conserved nucleotides were dark shaded.

4.2.4.4 Validation of reference genes for petunia Cu-Zn SOD gene expression

In order to select an appropriate internal control gene for normalizing the expression of the target gene (Cu-Zn SOD) using real-time RT-PCR, the expression variability of the most commonly used housekeeping genes, 18S rRNA and ß-actin genes were also evaluated in addition to GAPDH gene. The quantification curves of the four genes (*Figures 4.20 A, 4.22 A, 4.24 A* and *4.26 A)* showed that PCR amplification performed normally, with high plateau values and longer logarithmic phases. The melting curves obtained of the four genes (*Figures 4.20 B, 4.22 B, 4.24 B* and *4.26 B*) indicated that one specific product was generated in each PCR reaction. For both the target and reference genes, the expression levels represented by mean Ct value and changes of Ct value in control and ethanol-treated petunia petals are listed in *Table 4.1*. The effect of petal development and senescence on expression levels is also shown for water-treated petunia petals at day 0, 3 and 6.

Expression of target gene (Cu-Zn SOD) expression was significantly influenced by the ethanol treatment and petal developmental stage. On day 6, a 3.83 cycle difference of Ct value was observed between ethanol- and water-treated petunia petals while the water-treated petals had 3.08-cycle difference when compared to the day 0 petals. Similarly, GAPDH expression was also markedly affected by ethanol treatment and petal developmental stages. The difference between ethanol and control petals was 3.10 and that of control petals at day 0 and day 6 was 3.33. Although the ethanol treatment did not affect the changes of the Ct value of the actin gene, the Ct value did decrease significantly in the control petals since a 2.53 or 3.71 cycle difference was observed respectively between day 3 or day 6 and day 0. 18S rRNA expression was relatively stable and the Ct number varied slightly during the experimental period with a cycle difference of only 0.10 on day 3 and 0.17 on day 6 compared to that of day 0. Ethanol had little effect on expression level of 18S rRNA gene because the differences of Ct values between the ethanol- and water-treated petunia petals were minor, only 0.31 on day 3 and 0.64 on day 6. Since the changes of Ct number in the case 18S rRNA

expression were almost constant, independent of ethanol treatment and petal development during the post-harvest vase-life experiment, it was chosen as the reference gene for later quantification of target gene expression.

4.2.4.5 Regulation of gene expression by ethanol treatment

Fifty-fold diluted cDNA of petunia petals after treatment with water or 6% ethanol for different days was chosen as the templates for real-time quantitative PCR assays. For the target gene (Cu-Zn SOD) and reference genes (18S rRNA, GAPDH and actin) normal and typical PCR accumulation profiles were observed (*Figures 4.34 A, 4.35 A, 4.36 A* and *4.37 A*). The melting curves indicate that only one expected product was produced for every gene of interest in every sample (*Figures 4.34 B, 4.35 B, 4.35 6* and *4.37 B*). This was confirmed using the agarose gel electrophoresis showing the appropriate PCR products (*Plates 4.21* and *4.22*).

In this study, expression of the target gene and reference genes in petunia petals with respect to time and the ethanol treatment were presented as $(1+E)^{\Delta Ct}$, where day 0 or control group was accepted to be 1-fold expression of each gene and $\Delta Ct = Ct_{\text{day x}} - Ct_{\text{day 0}}$ or $Ct_{\text{control}} - Ct_{\text{ethanol}}$. The relative mRNA levels were expressed as the ratios of target gene expression to 18S rRNA expression.

During petal senescence the mean Ct value, which reveals expression of Cu-Zn SOD gene, increased in both ethanol- and water-treated petunia petals (*Table 4.1*). This indicates that down-regulation of Cu-Zn SOD gene expression was associated with petunia petal senescence. In control petunia petals, Cu-Zn SOD mRNA level calculated from the reaction efficiency and Ct value was decreased significantly to 67% on day 3 and 14% on day 6 when compared to that of day 0 (*Figure 4.38 A*). Similarly, if mRNA level was expressed as the ratio of Cu-Zn SOD mRNA level to 18S rRNA level, the decrease would be 77% on day 3 and 15% on day 6.

The values of amplified Cu-Zn SOD cDNA for ethanol-treated petals were compared with those of the water control in order to determine whether ethanol had an effect on the amount of Cu-Zn SOD mRNA quantified using real-time PCR. When compared to water-treated petals, there was slightly more than a 2-fold increase in mRNA level of Cu-Zn SOD after 3 days of ethanol treatment with or without normalization to expression of the internal reference gene, 18S rRNA gene. In both cases, the difference was not significant. However, on day 6, without normalization, about 20-fold up-regulation of Cu-Zn mRNA level in petunia petals was observed in the ethanol treatment (*Figure 4.38 B*). After normalization with reference gene expression, the up-regulation ratio was, on average, 55-fold.

The pattern of mRNA level of GAPDH was similar to that of Cu-Zn SOD. Firstly, a decreased expression of GAPDH gene was observed during petunia petal senescence. When compared to that of day 0, the expression of GAPDH gene did not vary much during the first three days (*Figure 4.39 A*). However, on day 6, it was significantly down-regulated to about 12% with or without normalization to 18S rRNA expression. Secondly, ethanol also significantly up-regulated the mRNA level of GAPDH by a factor of about 7.82. With normalization of the endogenous standard transcript, the exact relative expression ratio can be calculated as 16 on day 6 (*Figure 4.39 B).* Just like Cu-Zn SOD gene expression, there was no significant difference between the mRNA levels of GAPDH in ethanol- and water-treated petals on day 3.

The mRNA level of actin with or without normalization to 18S rRNA expression was significantly decreased in control petals from day 3 (*Figure 4.40 A*). However, ethanol had little influence on actin mRNA level since there were no significant differences in the mRNA levels between ethanol- and water-treated petunia petals during the experimental period (*Figure 4.40 B*).

Table 4. 1 Quantification of the expression level of the target gene (Cu-Zn SOD) and three reference genes in petunia petals after ethanol (6%) or water (control) treatment

Genes	Days after treatment	Treatments	Mean Ct value	ΔCt [a] ($C_{t\ control}$-$C_{\ t\ ethanol}$)	ΔCt [b] ($Ct_{\ day\ x}$-$C_{\ t\ day\ 0}$) (control petals)
Cu-Zn SOD	0		29.60 ± 0.45		
	3	Ethanol	29.11 ± 0.26		
	3	Water	30.24 ± 0.45	1.13 a	0.64 a
	6	Ethanol	28.85 ± 0.20		
	6	Water	32.68 ± 0.56	3.83 b	3.08 b
GAPDH	0		26.29 ± 0.14		
	3	Ethanol	25.73 ± 0.13		
	3	Water	26.35 ± 0.22	0.62 a	0.06 a
	6	Ethanol	26.52 ± 0.08		
	6	Water	29.62 ± 0.09	3.10 b	3.33 b
Actin	0		35.78 ± 0.52		
	3	Ethanol	38.28 ± 0.50		
	3	Water	38.31 ± 0.32	0.03 a	2.53 a
	6	Ethanol	39.93 ± 0.50		
	6	Water	39.49 ±0 .35	-0.45 a	3.71 b
18S rRNA	0		16.44 ± 0.81		
	3	Ethanol	16.23 ± 0.65		
	3	Water	16.54 ± 0.58	0.31 a	0.10 a
	6	Ethanol	17.25 ± 0.14		
	6	Water	16.61 ± 0.71	-0.64 a	0.17 a

Total RNA was isolated and reverse transcribed into cDNA for real-time quantitative amplification. The Ct numbers are shown as means ± SE values. ΔCt [a]: the Ct of ethanol-treated petals normalized to that of control petals; ΔCt [b]: the Ct value of different days normalized to that of day 0 in water-treated petals. Different letters on the same particular parameter indicate statistical difference at P=0.05.

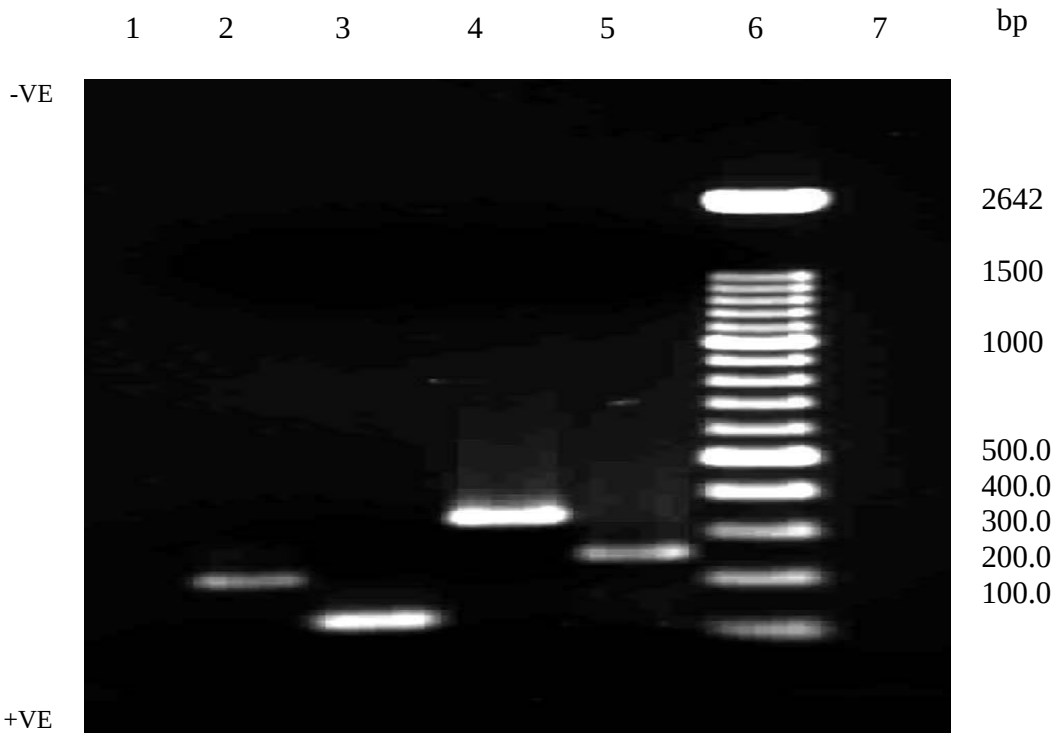


Plate 4. 21 Agarose gel electrophoresis of the products from real-time PCR amplification of Cu-Zn SOD, GAPDH, actin and 18S RNA using petunia petal cDNA as the template

Gene fragments in lane 1-4: actin **(**Lane 1); GAPDH (Lane 2); 18S rRNA (Lanes 3); Cu-Zn SOD (Lane 4); Lane 5: 1 kb DNA marker. 2% (w/v) agarose was used for gel electrophoresis (80 V for 1.5 hours).

The expected sizes of the Cu-Zn SOD, 18S rRNA, GAPDH and actin amplicons were about 250, 330, 147 and 185 base pairs, respectively. The petals used for RNA isolation were freshly harvested and without any treatment.

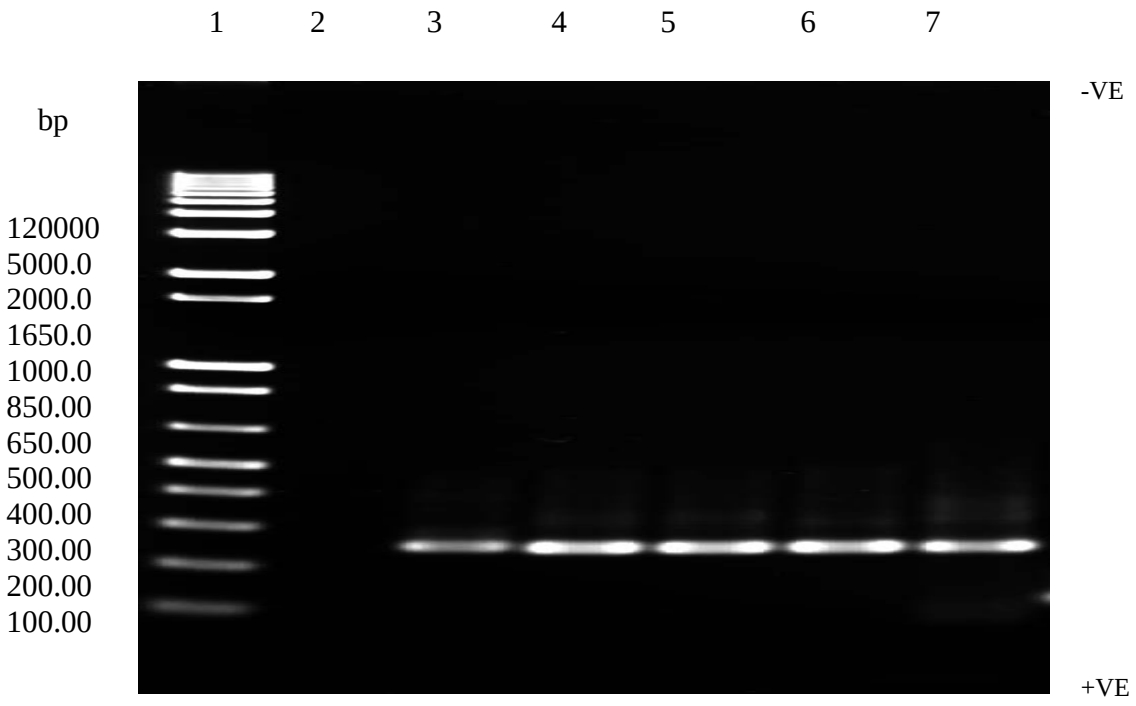


Plate 4. 22 Agarose gel electrophoresis of the products from real-time PCR amplification of Cu-Zn SOD cDNA reverse transcribed from RNA extracted from petunia petals at different days after ethanol (6%) or water treatment

Lane 1: 100 kb markers; Lane 2: negative control (absence of template); Lanes 3 and 5: a gene fragment of Cu-Zn SOD on day 6 and 3 respectively following water treatment; Lanes 4 and 6: the same as Lanes 3 and 5 following ethanol treatment; Lane 7: fresh petals without any treatment. Agarose (2%, w/v) was used for gel electrophoresis (80 V for 1.5 hours).

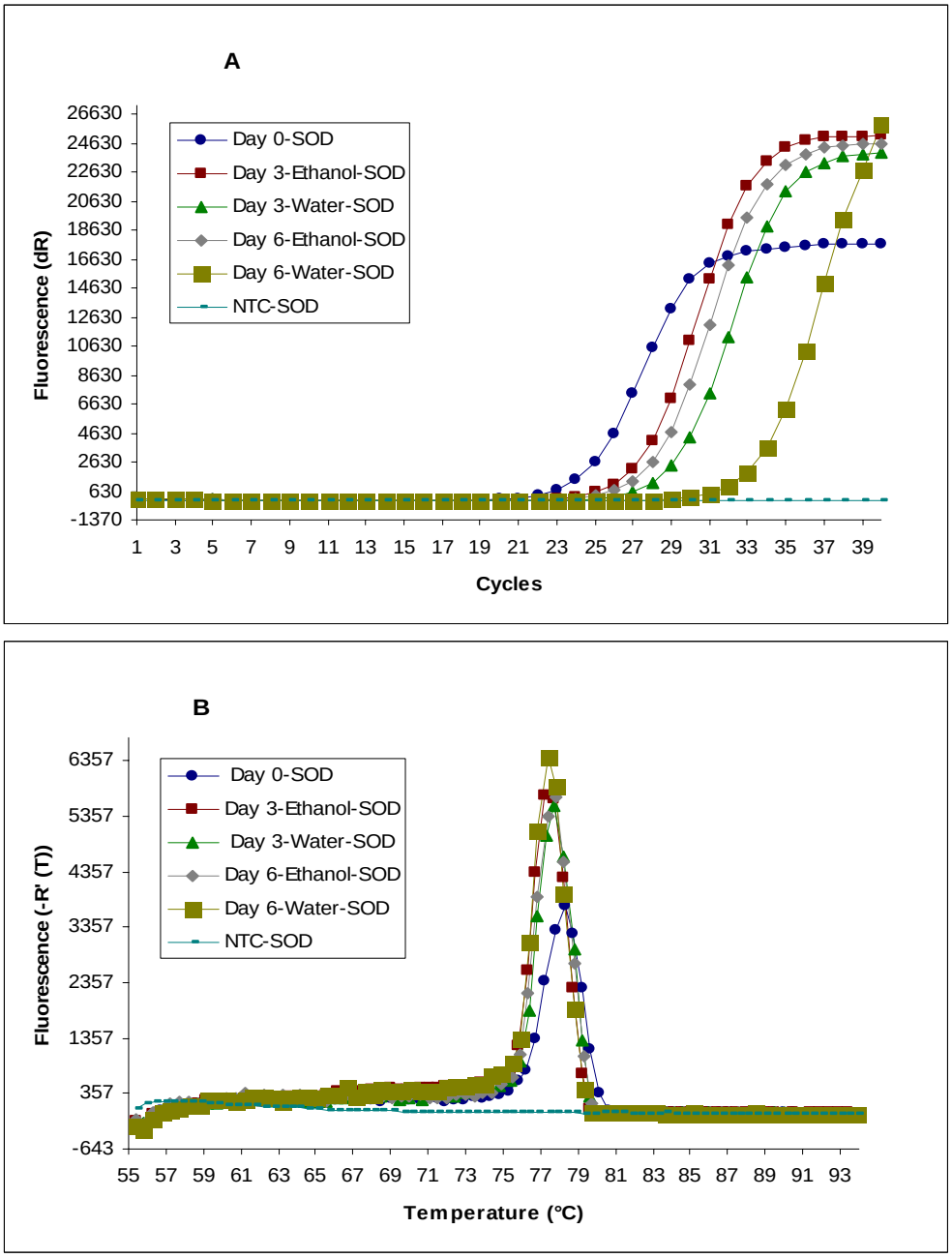


Figure 4. 34 Rreal-time PCR amplification of a Cu-Zn SOD gene fragment at different times following ethanol (6%) or water treatment of petunia petals

Upper panel (A): quantification curves; lower panel (B): melting curves; NTC: no template control.

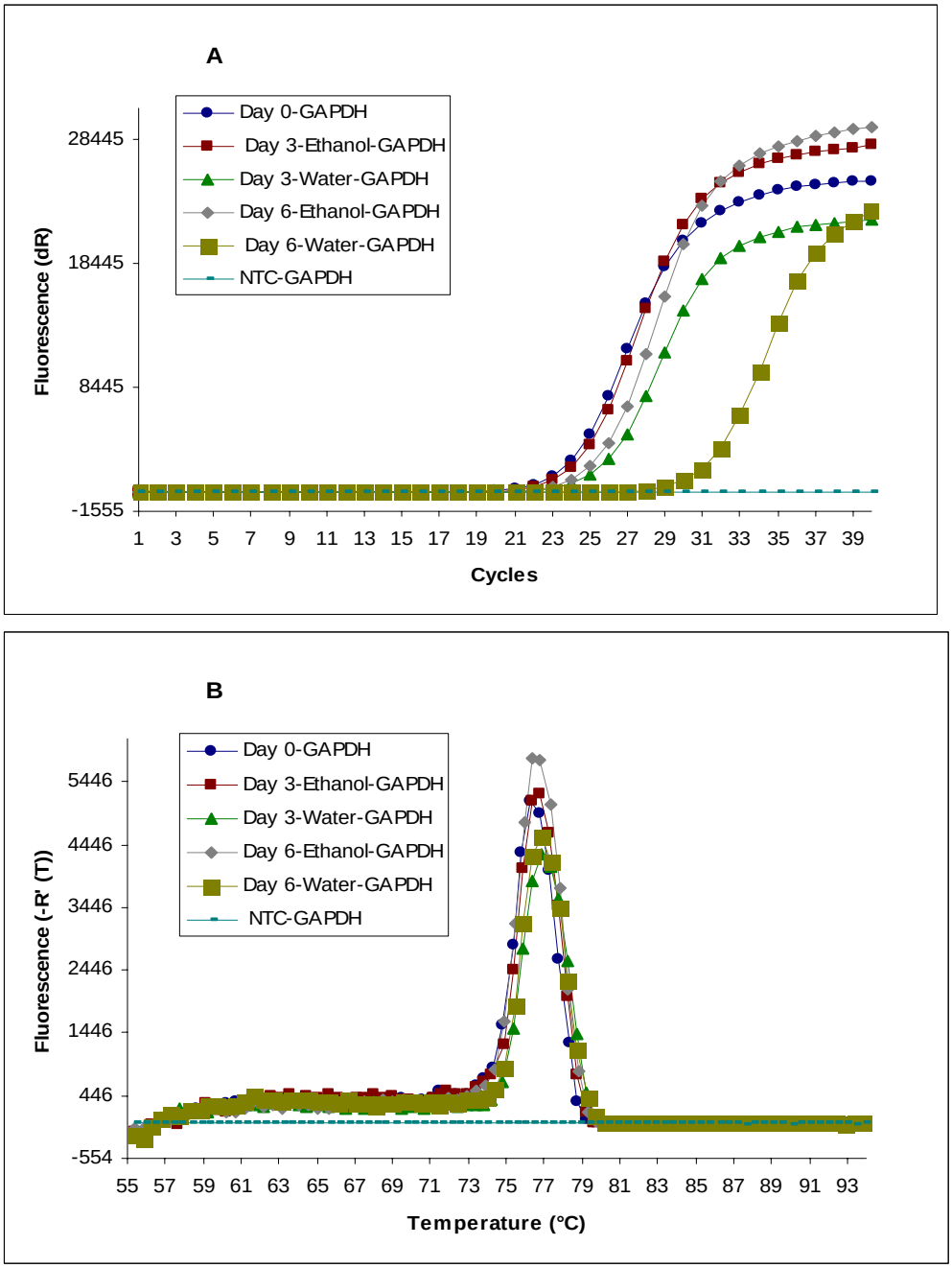


Figure 4. 35 Real-time PCR amplification of a GAPDH gene fragment at different times following ethanol (6%) or water treatment of petunia petals

Upper panel (A): quantification curves; lower panel (B): melting curves; NTC: no template control.

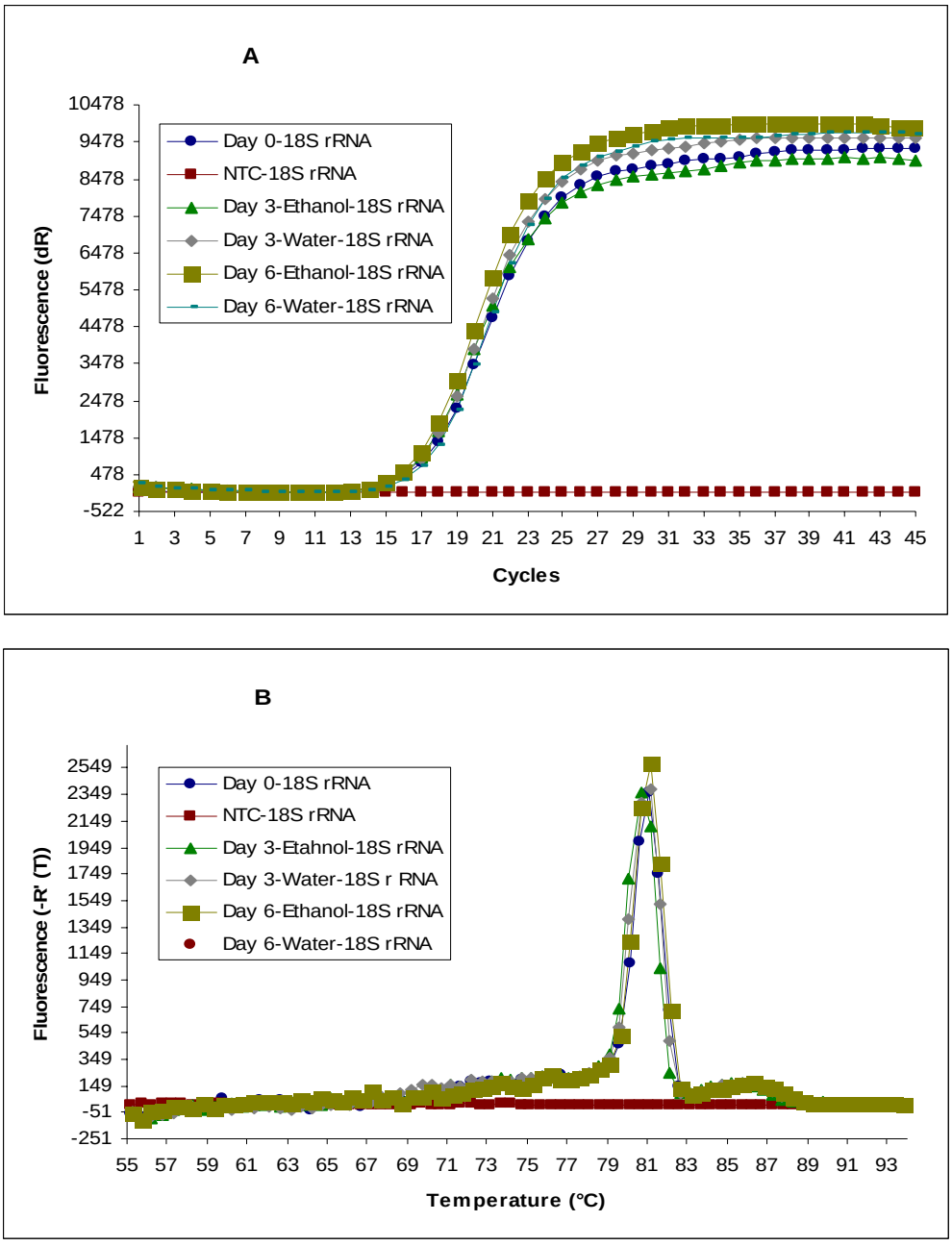


Figure 4. 36 Real-time PCR amplification of a 18S rRNA gene fragment at different times following ethanol (6%) or water treatment of petunia petals

Upper panel (A): quantification curves; lower panel (B): melting curves; NTC: no template control.

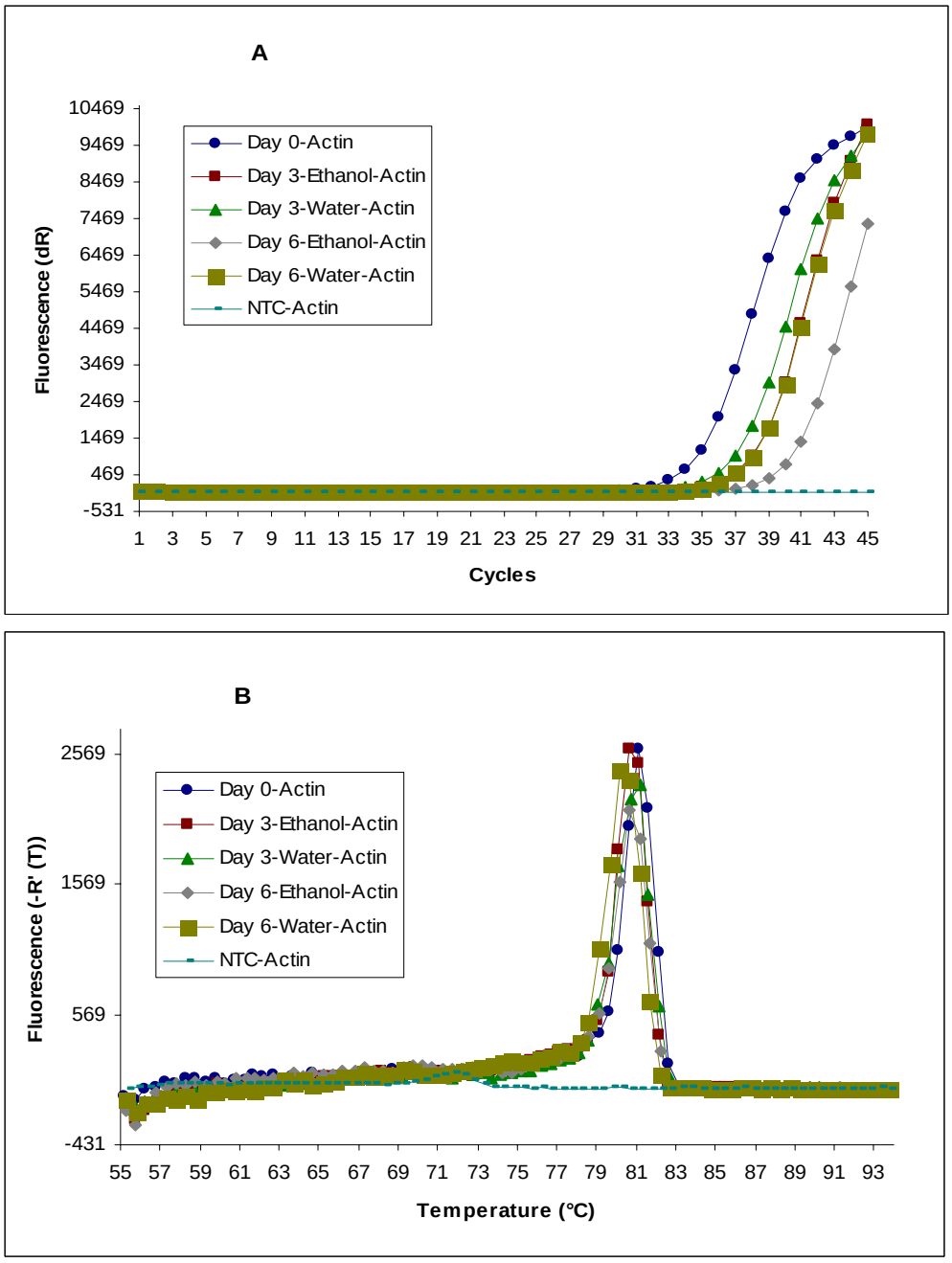


Figure 4. 37 Real-time PCR amplification of an actin gene fragment at different times following ethanol (6%) or water treatment of petunia petals

Upper panel (A): quantification curves; lower panel (B): melting curves; NTC: no template control.

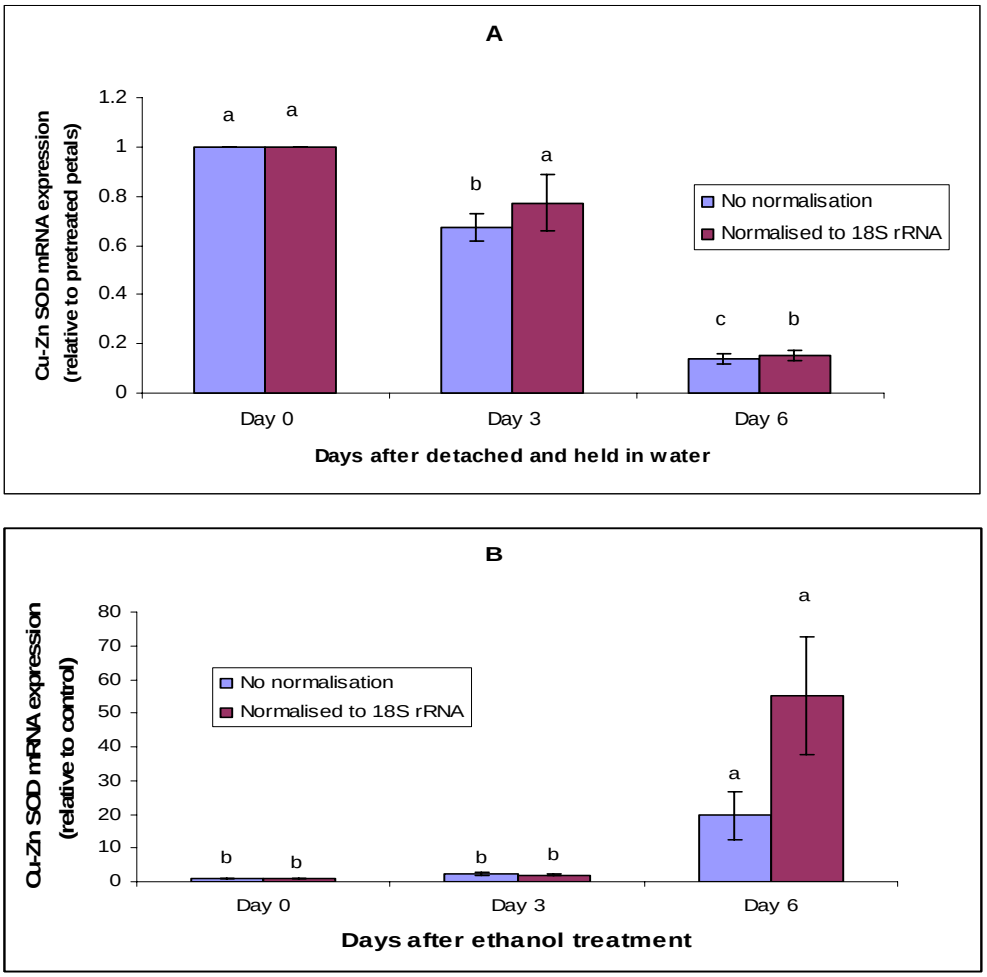


Figure 4. 38 Effect of ethanol treatment on Cu-Zn SOD mRNA level relative to 18S rRNA level in petunia petals

After total RNA was isolated and reverse transcribed, real-time amplification of Cu-Zn SOD cDNA was performed. Results are means ± SE of three determinations from three individual extracts. mRNA level was expressed as $(1+E)_{\text{Cu-Zn SOD}}^{\Delta Ct_{\text{Cu-Zn SOD}}}$ (not normalized) or the ratio of Cu-Zn SOD abundance relative to 18S rRNA expression calculated using the formula: ratio = $(1+E)_{\text{Cu-Zn SOD}}^{\Delta Ct_{\text{Cu-Zn SOD}}}$ / $(1+E)_{\text{internal control}}^{\Delta Ct_{\text{internal control}}}$; ΔCt: the differences of Ct values between control and ethanol-treated petunia petals or between a particular day and day 0 petals; E: real-time PCR reaction efficiency. Bars with different letters indicate that the results are significantly different using Tukey's method ($p < 0.05$).

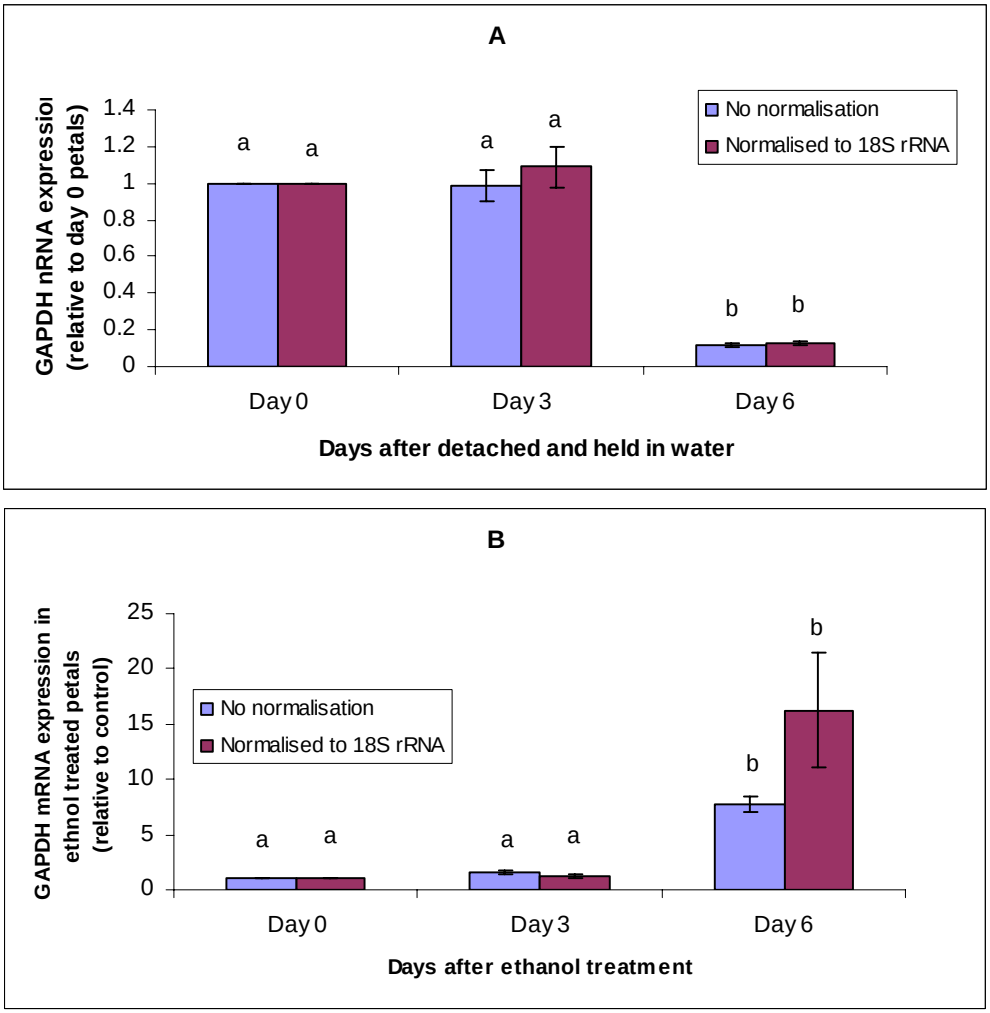


Figure 4. 39 Effect of ethanol treatment on GAPDH mRNA level relative to 18S rRNA level in petunia petals

After total RNA was isolated and reverse transcribed, real-time amplification of GAPDH cDNA was performed. Results are means ± SE of three determinations from two individual extracts. mRNA level was expressed as $(1+E)_{GAPDH}^{\Delta Ct_{GAPDH}}$ (not normalized) or the ratio of GAPDH abundance relative to18S rRNA expression calculated using the formula: ratio = $(1+E)_{GAPDH}^{\Delta Ct_{GAPDH}} / (1+E)_{internal\ control}^{\Delta Ct_{internal\ control;}}$ ΔCt: the differences of Ct values between control and ethanol treated petunia petals or between a particular day and day 0 petals; E: real-time PCR reaction efficiency. Bars with different letters indicate that the results are significantly different using Tukey's method ($p < 0.05$).

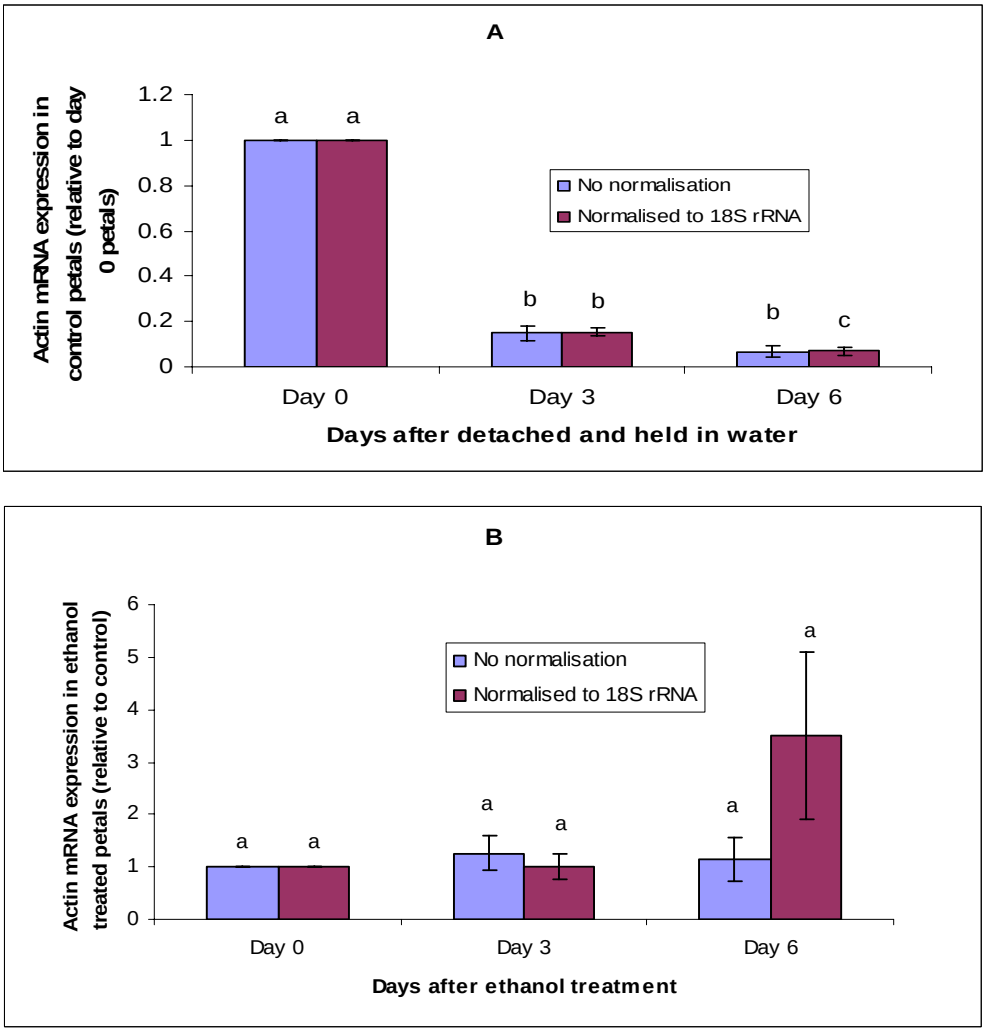


Figure 4. 40 Effect of ethanol treatment on actin mRNA level relative to 18S rRNA level in petunia petals

After total RNA was isolated and reverse transcribed, real-time amplification of actin cDNA was performed. Results are means ± SE of three determinations from two individual extracts. mRNA level was expressed as $(1+E)_{actin}^{\Delta Ct_{actin}}$ (not normalized) or the ratio of actin abundance relative to18S rRNA expression calculated using the formula: ratio = $(1+E)_{actin}^{\Delta Ct_{actin}} / (1+E)_{internal\ control}^{\Delta Ct_{internal\ control;}}$ ΔCt: the differences of Ct values between control and ethanol-treated petunia petals or between a particular day and day 0 petals; E: real-time PCR reaction efficiency. Bars with different letters indicate that the results are significantly different using Tukey's method ($p < 0.05$).

4.3 Discussion

4.3.1 Oxidative events associated with development and senescence of petunia petals

4.3.1.1 Changes in antioxidants

Generally, elevated antioxidative enzyme defence in a plant could play a significant role in tolerance or adaptation to various abiotic stresses (Barna and Pogany, 2001; Nagata et al., 2003). For example, higher levels of antioxidant metabolites and antioxidative enzymes were observed during the dormant season in eastern white pine needles. The activities of antioxidative enzymes were increased in turf leaves to adapt to increasing temperatures in the early summer (Anderson et al., 1992; Ervin et al., 2004). In flowers, a coordinated regulation of antioxidative defence is also essential for petal development and senescence. Data presented here shows that many isoforms of SOD, AP and POD were detected on the activity gels of crude petunia petal extracts (*Plate 4.4*). Petals possess SOD, POD and AP to metabolize $O_2^{\cdot-}$ and H_2O_2. Following petunia petal development, POD activity increased to a maximum before flower senescence and then decreased until the last stage of petal life. A similar finding was from a study on maize leaf senescence (Prochazkova et al., 2001). Increase of POD activity was found in four other senescing systems: daylily, chrysanthemum, cucumber and lupin cotyledons (Bartoli et al., 1995; Feng et al., 2003; Hernandez et al., 2002; Panavas and Rubinstein, 1998a).

Another well-known reaction involving the reduction of H_2O_2 is by AP, with the consequence of ASC being oxidised to DHA. In higher plants, AP isozymes are distributed in five cellular compartments: stromal AP, thylakoid membrane-bound AP in chloroplast, microbody membrane-bound AP, cytosolic AP and mitochondrial membrane-bound forms (Shigeoka et al., 2002). The trend of AP activity in petunia petals was that of a clear and gradual increase first, and then a decrease. This was similar to the

change of AP activity during senescence of tobacco and maize leaves (Dertinger et al., 2003; Prochazkova et al., 2001). It was reported that a significant increase of AP activity was associated with the initial stage of *Chrysanthemum* petal senescence (Bartoli et al., 1995). However, a drop in AP activity was observed during senescence of daylily flower, lupin and cucumber cotyledons (Feng et al., 2003; Hernandez et al., 1992; Panavas and Rubinstein, 1998a).

A very high SOD activity was associated with the development of petunia petals. In particular, the high activity of S_2 (the main component of SOD isozymes) did not alter dramatically throughout petal development and early senescence, suggesting that S_2 might play an important function to eliminate the superoxide produced in petals. As there were visible signs of petal senescence, the activity of other SOD isoforms was found to decrease (*Plate 4.1*). This pattern of changes in SOD isoforms is consistent with that found during senescence of detached cucumber cotyledons (Feng et al., 2003). However, it is different from other reports on senescence of rose flowers, tobacco and maize leaves where an early increase of SOD activity and a later drop was found (Dertinger et al., 2003; Prochazkova et al., 2001; Sun et al., 2004). The increased SOD activity in specific parts such as the top of the petunia petal (that senesced earlier) may be masked by the decreased activity of lower petal parts (that senesced later).

The response of antioxidant systems to increasing oxidative stress during senescence does not appear to be consistent between plants and different antioxidant enzymes (Hodges et al., 2001). H_2O_2-utilizing enzymes play specific roles in the elimination of a high level of hydrogen peroxide and peroxide radicals. In petunia, the higher activities of AP and POD at early stages might be related to regulation of signal molecules such as H_2O_2. The later decrease of antioxidant enzyme levels may suggest that more enzymes are being inactivated and damaged. Suppression of AP in transgenic antisense tobacco plays a key role in elevating cellular H_2O_2 levels (Mittler et al., 1999). The reactivation of AP activity in *Arabidopsis*, at later developmental stages, was associated with a decline in lipid peroxidation (Ye et al., 2000). The process of peroxidation, initiated by superoxide and its derivative ($OH^{\cdot}$), of an adjacent unsaturated fatty acid can continue as

a chain reaction as long as an adjacent unsaturated fatty acid is available. This eventually will influence cell death and lead to plant senescence.

4.3.1.2 Changes in the levels of ROS during petunia petal development and senescence

The levels of ROS in a plant tissue are dependent on the status of ROS generation and removal. The accumulation of ROS in petals during senescence possibly indicates an elevated generation of radicals and / or a loss of the antioxidant capacity (Zimmermann and Zentgraf, 2004). Areas of formazan deposits might indicate that rates of superoxide and hydrogen peroxide production in these areas of the plant tissue had become significantly greater than those of detoxification. Appearance of more purple formazan production and brown pigment polymerization in control petunia petals than in other treatments suggests that senescence of petunia petals was accompanied by an accumulation of these ROS. Similar results were reported on the senescence of several plant tissues (Feng et al., 2003; Prochazkova et al., 2001; Rubio et al., 2004).

The pattern of superoxide accumulation is directly related to the changes of SOD activity in petal tissues. During petunia petal development, superoxide was detectable at Stage PS. More appeared at Stage HS but its level appeared to decrease at the final Stage SF (*Plate 4.5*). This correlates with a lower level of SOD activity found in the petals at the final stage of senescence. Higher SOD activities in developmental petals might enable them to keep superoxide to a low level. Therefore, less superoxide was found at earlier developmental stages such as BF, HF and OF using the imaging method.

Accumulation of hydrogen peroxide is also correlated with the appropriate antioxidative enzyme activities during petunia petal development and senescence. The imaging method used showed that there was a low level of hydrogen peroxide in young petunia petals. This might possibly be related to a higher AP activity.

At the last Stage HS, the decrease in activities of AP and SOD may be related to the relatively large amounts of superoxide and hydrogen peroxide detected in petunia petals. ROS production could far exceed the endogenous ROS scavenging capacity due to a combination of lower antioxidant defence and an equal or even higher level of ROS production.

Although in this study only accumulation of ROS such as $O_2^{\cdot -}$ and / or H_2O_2 was observed during petunia petal senescence, the levels of other ROS such as lipid peroxidation products could also increase (Leutner et al., 2001). The increased ROS levels in petunia petals during senescence could disturb cellular redox states. For example, there may be a concomitant reduction of unsaturated fatty acids and reduced glutathione with an increase in the concentration of oxidized forms of glutathione (Barata et al., 2005). This imbalance between ROS generation and scavenging system will result in oxidative stress in the petals. Subsequently, oxidative stress led to oxidative damage, inactivation, and degradation of antioxidative defense enzymes, including the associated proteins, mRNAs and DNA. Lipid peroxidation would also be promoted. Leakage and degradation of cell membrane eventually lead to tissue senescence (Evans et al., 1999; Hernandez et al., 2002).

4.3.2 Anti-senescence effect of ethanol on petunia petals

4.3.2.1 Effect of ethanol on levels of ROS

Delaying senescence in plants is not always linked to increased activities of antioxidative enzymes. Slower leaf senescence in mutants of Arabidopsis was associated with similar or even lower antioxidative enzyme activities when compared to the wild type (Woo et al., 2004).

There are many reports on the anti-senescence effect of free radical scavengers in animals (Liu et al., 1998; Okatani et al., 2002a; Okatani et al., 2002b; Wang et al., 2000). In cut

carnation, 3, 4, 5-trichlophenol can prolong vase-life of carnation and delay breakdown of fatty acids of polar lipids, loss of membrane integrity and massive peroxide production (Paulin, 1986).

The results obtained in this study showed that ethanol, as a free radical scavenger, could delay petunia petal senescence by the regulation of ROS formation and accumulation. This could subsequently limit the quantity of the extremely reactive oxidant, hydroxyl radical (·OH), and general membrane lipid peroxidation. A previous study showed that lipid peroxidation during leaf senescence was inhibited by ethanol treatment (Dhindsa et al., 1982). However, they found that ethanol treatment was unable to prevent SOD activity decline in leaves. In petunia, the higher activities of AP and SOD in ethanol-treated petunia petals are possibly related to the inhibition by ethanol of ROS-associated damage to these antioxidative enzymes.

4.3.2.2 Effect of ethanol on antioxidative enzyme activities

In this study, the anti-senescence effect of ethanol in petunia might be related to an enhanced adaptability to senescence-associated oxidative stress in terms of higher levels of antioxidative enzymes than in water-treated petals. Both test-tube enzyme assays and isozyme analyses following non-denaturing polyacrylamide gel electrophoresis support this. This is consistent with the report on broccoli showing that higher activities of peroxidise and superoxide dismutase were maintained following ethanol treatment when compared to the control treatment (Han et al., 2006a).

The higher levels of the isozymes SOD_2 and SOD_4, two of the predominant Fe-SOD forms in petunia petals, appeared to be of sufficient magnitude to account for the increased levels of total SOD activity in ethanol-treated petunia petals. They could be involved in reducing ROS level in ethanol-treated petals.

No enzymatic mechanism is known in cells to eliminate ·OH, which is a highly reactive agent with all biological molecules including DNA, lipid and carbohydrate, and amino acid residues of some important proteins and / or enzymes (Vranova et al., 2002). ·OH can reduce SOD activity by inducing fragmentation of Cu-Zn SOD by oxidizing His-118 at the active site of the enzyme to 2-oxo-His (Casano et al., 1997). The excess production of this highly reactive ROS directly threatens the life of a cell and eventually leads to cell death. In this study, ethanol, as a hydroxyl scavenger, can inhibit ROS accumulation in ethanol-treated petunia petals. This could at least partially protect the antioxidative defense enzymes from inactivation and degradation by ROS. Therefore, more antioxidative enzyme activity is retained in the ethanol-treated than control petals. This lower capacity of free radical scavenging system may contribute to the shorter vase life of control petunia petals.

4.3.2.3 Effect of ethanol on antioxidant gene expression

4.3.2.3.1 Factors affecting quantitative real-time RT-PCR assays

Ethanol plays remarkable roles in retarding the decrease of the activities of both Fe SOD (S_2 and S_4) and Mn SOD (S_1). However, the activity of the Cu-Zn SOD isozyme in petunia petals was very low. It was difficult to detect if there is a difference between ethanol and water treatments in Cu-Zn SOD isozyme activity on PAGE gels. Therefore, quantifying the expression of this gene at transcription level would be a better alternative to resolve this problem. A PCR based method for gene expression analysis is supposed to be suitable due to its high sensitivity and analytical power. However, the conventional PCR technique is less competitive for this purpose because the detection based on endpoint accumulation of a PCR product can only be qualitative or semi-quantitative. Real-time PCR can accurately quantify a PCR product as it collects data during the exponential phase of an amplification curve. At this time the PCR reagents are not limited and the amplification variations due to other factors are minimized. It is also very efficient in analyzing a high number of target genes at the same time using minute

quantity of RNA due to its broad detecting range and high sensitivity (Gachon et al., 2004).

In this study, a two-step SYBR Green I based real-time PCR protocol was successfully used to assess Cu-Zn SOD gene expression in petunia petals treated with water or ethanol. The first step involved RNA isolation and cDNA synthesis and the second step was real-time PCR with gene specific primers. In order to maximize the reproducibility of detecting the difference between ethanol and water treatments, it is critical to reduce the experimental error in every step such as RNA extraction, cDNA synthesis, real time PCR analysis including different PCR runs to the minimum level.

One of the most important factors for a successful RT- PCR assay is the quality of isolated RNA. The approximately 1:2 ratio of the 18S and 28S ribosomal RNA band pattern observed in this study indicated a good quality of the RNAs isolated from most petunia petal samples since more than 90% of the total cellular RNA measured using spectrophotometry was reported to be ribosomal RNA (Glare et al., 2002). The appropriate annealing temperature, and the most suitable primer pairs (F_1R_2) for Cu-Zn SOD gene were also determined after testing a range of the real-time PCR conditions.

4.3.2.3.2 Validation of quantitative real-time RT-PCR results

Typical standard curves were obtained for real-time PCR amplification of all genes of interest from petunia petal cDNA. As shown in *Figures 4.21, 23, 25,* and *4.27,* a high log-linearity at four orders of magnitude (1, 10, 100, 1000, and 10000-fold) was evident for most of the genes of interest. The correlation coefficients were approximately equal to or greater than 0.99. The efficiency of the PCR reaction was typically greater than 85%. This indicates that the quantification of gene expression used in this study is reliable. The specificity of SYBR Green detection of PCR products is also verified by consistent melting curve analysis and agarose gel electrophoresis.

Several factors may affect the accurate quantification of gene expression. For example, a primer-dimer could be formed and genomic DNAs might be a contaminant in the RNA sample. Two-step RT PCR was used since it is supposed to be less prone to problems related to production of primer-dimer artefacts and genomic contamination (Kreuzer et al., 1999; Vandesompele et al., 2002). The pseudogene sequences in genomic DNA are sometimes co-amplified by primers that were expected to be specific for cDNA templates (Kreuzer et al., 1999). It was reported that many ingredients of a reverse transcription reaction could inhibit the subsequent real-time PCR reaction. In this study, undiluted cDNAs did exhibit some inhibitory effects on real-time PCR reactions. In the case of all the eight tested primer-pairs for Cu-Zn SOD and GAPDH genes, a non-specific PCR product was generated in addition to the main amplicon when undiluted or 10-fold diluted cDNA was used as a template. Interestingly, when cDNA was diluted to 50-fold or more, this non-specific product was no longer detected, suggesting that the non-specific product is possibly due to a high concentration of RT components during cDNA synthesis.

Another possible reason may be genomic DNA contamination. In order to overcome this problem, some measures were taken by other researchers. The measures include carefully selecting primer biding sites in order to span at least one intron to eliminate any PCR-amplified fragments of genomic DNA (Xie et al., 2002). In our case, before cDNAs were synthesized, one μg of different RNA samples was subsequently incubated with RNase-free DNAase (M0303S**)** for 30 min at 37°C in order to remove possible contaminating genomic DNA. Although occasionally the same specific amplification product appeared in the control tubes (in the absence of RT), by analyzing the amplification curves and melting curves, they were negligible since generally they appeared 8-10 cycles later than the target products. DNase was used successfully and greatly minimized the problems of pseudogenes and non-specific DNA amplification.

Primer-dimers occasionally appeared in the negative control (absence of RT or template) in this study. Generally, when cDNAs were added to the reaction mixture, primer-dimers disappeared during 45 PCR cycles of PCR amplification for the target genes. The

addition of a short, high temperature incubation step (74˚C for 10 seconds) after the elongation step conferred further specificity to the fluorescence signal and accurate amplification values since the melting temperature of these primer-dimers was generally lower than 74˚C. In order to eliminate the appearance of primer-dimer formation in control tubes, more experiments in the future to determine optimal primer concentration would be helpful.

4.3.2.3.3 Evaluation of a housekeeping gene to be used as an internal control

Normalization of gene expression data is essential for accurate interpretation of the experimental results. Microplate-based fluorometric assays using the PicoGreen reagent has been used to eliminate the inherent variability associated with differences in the efficiency of a reverse transcription reaction (Cheung et al., 2004). Although there is no universal method for normalizing gene expression data generated using real-time PCR, the common practice is to use a reference gene to correct experimental errors due to differences in RNA and cDNA load, tissue mass, cell number, experimental treatments or RNA extraction and cDNA synthesis efficiency. Identification of a valid reference gene for data normalization is crucial in real-time PCR experiments. Ideal internal controls should have stable expression levels under different developmental stages and experimental conditions

GAPDH gene has been used in various studies as a reference gene because its function as a glycolytic intermediate is expected to be present in all cells and exhibiting minimal modulation. It is reported to be a stable and suitable reference gene for a range of experimental systems including one apoptotic system (Kumar and Joyner, 2003; Matyas et al., 1999 ; Ullmannova and Haskovec, 2003). However, expression of GAPDH gene was also reported to be increased during fetal organ development and therefore was not suitable as a reference gene (Al-Bader and Al-Sarraf, 2005). This possibly indicates its involvement in other non-glycolytic activities as well. Although it is not perfect, GAPDH gene has been reported to be one of the most popular reference genes used in real-time PCR investigations (Sturzenbaum and Kille, 2001). Therefore, it was used initially in this

study. However, GAPDH gene expression was found to decrease during petunia petal senescence and ethanol treatment influences this as well. Therefore, GAPDH gene is not considered as a suitable internal control to quantify Cu-Zn SOD gene expression in petunia petals. However, during petal senescence a decrease in the expression of GAPDH gene was relatively slower than that of Cu-Zn SOD. In addition, with or without the normalization to expression of internal control, ethanol upregulated expression of GAPDH gene by a factor of 7.82 or 16 respectively, which are much less than that of Cu-Zn SOD.

It is a good practice to investigate several housekeeping genes at the same time and select the most suitable one to be used as an internal control. Furthermore, multiple housekeeping genes may be required in the same experimental system for accurate normalization of experimental data (Vandesompele et al., 2002). Therefore, both actin and 18S rRNA genes were used in the current study as well. Similar to GAPDH gene, expression of actin gene varied remarkably during petunia petal senescence and it was subsequently considered not suitable as an internal control either. However, expression level of 18S rRNA gene was independent of the ethanol treatment and petal development. It was therefore ideal as an internal reference gene under the conditions of this study.

4.3.2.3.4 Regulation of Cu-Zn SOD gene expression by ethanol

Antioxidative copper-zinc superoxide dismutase (Cu-Zn SOD) plays an important role in scavenging superoxide anion radicals. It is possibly involved in the process of senescence. It has been found that the absence of Cu-Zn SOD in mice results in age-related degeneration such as hearing loss at an earlier age (Coling et al., 2003; Keithley et al., 2005). In this study, we showed that petunia petal senescence was also associated with a decrease in the transcription of Cu-Zn SOD gene expression. This is consistent with the report of a clear decrease of cytoplasmic Cu-Zn SOD gene expression in leaves of tobacco plants during senescence (Obregon et al., 2001).

Ethanol has also been found to upregulate transcription of a few genes during the process of aging or senescence. It could reverse the age-related hypo-expression of two nuclear receptors and two target genes involved in synaptic plasticity, neurogranin and neuromodulin in mice brain (Boucheron et al., 2006). Chronic ethanol consumption also restores the ageing-associated decline in the levels of mRNA of neurogranin (Ng), a Ca^{2+}-sensitive calmodulin-binding neuron-specific protein (Krazem et al., 2003). It is interesting that senescence-associated down-regulation of Cu-Zn SOD mRNA level in petunia petals was retarded by ethanol treatment. This suggests that ethanol treatment could enhance the antioxidant ability in petunia petals to scavenge free radicals.

There is an extensive overlap between senescence and stress responses since many of transcription factor genes that are induced during senescence are also induced by various stressors (Guo and Gan, 2005). A common factor involved in different stress responses and senescence is increased levels of reactive oxygen species. Since ROS have been proposed to be common signals, they may mediate the regulation of the same group of genes during senescence as well as in response to stress conditions.

Cu-Zn SOD has been used as a marker of oxidative stress since increased levels of Cu-Zn SOD occurred during oxidative stress in various studies (De-Lustig et al., 1993). Transgenic tobacco and potato that overexpressed a Cu-Zn SOD gene had a greater resistance to ozone (Pitcher and Zilinskas, 1996) and methyl-viologen (MV)-mediated oxidative stress (Gupta et al., 1993; Perl et al., 1993). However, no detectable increase in the protection of photosynthetic activity was found in the transgenic tobacco plants with a high expression level of a chloroplastic Cu-Zn SOD gene from petunia hybrid when these plants were exposed to MV (Tepperman and Dunsmuir, 1990). There is no evident reduction in the transgenic plants of symptom development after ozone fumigation as well (Pitcher et al., 1991).

The enhanced Cu-Zn SOD gene expression in ethanol-treated petals indicates a higher ability of petals to scavenge superoxide radical and/or a low level superoxide in the petals. On the contrary, the low level of Cu-Zn-SOD gene expression in control petals

might indicate an increased oxidative stress, which could render them particularly susceptible to oxidative damage and eventually lead to earlier petal senescence.

4.3.2.3.5 Effect of ethanol on GAPDH gene expression

It has been reported that in plants, H_2O_2 as a signal molecule, is involved in regulation of various processes including gene expression and programmed cell death (Hancock et al., 2005). In low concentrations, H_2O_2 serves as a signaling molecule to regulate the free radical scavenging system to coordinate flower senescence. However, at higher concentrations, it may exert toxic effects and poses a constant oxidative threat to cellular structures and processes, which is one of the factors responsible for the last stage of senescence and cell death. For example, H_2O_2-induced leaf senescence in rice was associated with an increase in lipid peroxidation indicated by an increased malondialdehyde level (Luijten et al., 1998). In tobacco disrupting mitochondrial function by H_2O_2 resulted in a decrease in the rates of electron transport, cellular ATP levels and a rapid rise in intracellular ROS accumulation (Maxwell et al., 2002).

GAPDH is recognized as an important protein that is sensitive to oxidative stress since its activity depends on the thioredox-mediated reduction of intromolecular disulfide bond to thiol groups. It might play a role in mediating ROS signaling in plants (Hancock et al., 2005). Expression of GAPDH has been reported to increase upon oxidative stress resulting from inhibition of endogenous Cu-Zn SOD (Ito et al., 1996). Similarly the transcription of GAPDH gene was specifically increased during programmed cell death (Sirover, 1997). Transcription of one cloned gene named as LSC540 was increased during senescence, and its possible function has been reported to be glyceraldehyde-3-phosphate dehydrogenase (Buchanan et al., 2000). It would be interesting to study expression of GAPDH gene in petunia petals since GAPDH might be involved in the process of petal senescence.

It has been found that hydrogen peroxide can directly inactivate cytosolic glyceraldehyde

3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) by oxidation of the thiol group *in vitro*, and this inhibition is reversible by the subsequent addition of reductants such as reduced glutathione (Hancock et al., 2005; Zaffagnini et al., 2007). Thus, the observed senescence-associated down-regulation of GAPDH gene expression is possibly related to an increased level of hydrogen peroxide during senescence in petunia petals. We also found that prolonging vase life of petunia petals by ethanol treatment was correlated with promotion of GAPDH gene expression. GAPDH has been found to be able to control generation of H_2O_2 by heat shock, which in turn suppresses cell death in yeast and plant cells (Baek et al., 2008). Therefore, the higher level of GAPDH expression in ethanol-treated petals indirectly suggests a low level of hydrogen peroxide in ethanol-treated petunia petals. This is consistent with the results in hydrogen peroxide determination in section 4.2.2.7.

Moreover GAPDH, a key classical enzyme in the control of glycolysis, is involved in basic metabolic processes including energy production, and has important housekeeping functions (Baek et al., 2008). The enhancement of general metabolic processes may be involved in the regulation of petal senescence by ethanol.

Taking together, our results indicate that the anti-senescence effect of ethanol might contribute to reduced oxidative stress in petunia petals by inhibiting reactive oxygen production or accumulation. The observed improved antioxidant status in ethanol-treated petals together with decreased levels of ROS is consistent with the oxidative stress theory of senescence. This implies that the optimal pro / antioxidant balance is critical for the anti-senescence effect of ethanol to control successfully petal vase life and to extend petal longevity. The decreased vase life of control petunia petals might result from their antioxidative enzyme activities not high enough to scavenge increased ROS production. The beneficial effect of ethanol on petunia flower vase life was probably to reduce deteriorative oxidative damages that could occur in plastids and other parts of a petal cell associated with advanced stages of senescence.

In summary, although the exact mechanisms for petal senescence are still not clear, the observations reported in this study may partially explain the possible anti-senescence mechanism of ethanol in petunia. One mechanism is an early inhibition of ROS formation and / or accumulation in petunia petals. This could limit the development of oxidative stress and damage, including the damage of antioxidative defense enzymes.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

5.1 Major findings of this study

Senescence is a complex process involving multiple organs, tissues and events. The process of oxidative and antioxidative defense is probably one of the various pathways involved. The main findings of this study on this topic are as follow:

- Pulsing cut gentian stems with GA_3 (1-100 μM) and sucrose (3%) delayed the time for the first flower on the stems to show signs of senescence. However, a significant effect on the time of the first flower to open was not observed. In the detached single flower system, the changes in petal weight and electrolyte leakage were significantly suppressed particularly when treated at the unopend stages. Petal discoloration was significantly retarded by GA_3 application. The decrease in photosynthetic pigment (chlorophylls and carotenoids) content in gentian petals during senescence was slowed down by GA_3. Activities of AP and SOD but not PDD in the GA_3-treated petals were significantly higher than those of the control.

- Ultrastructural changes of gentian petals were observed during development and senescence. Particularly senescence-associated features such as an increase in the

number and size of plastoglobuli, chloroplast swelling, large lipid accumulation; degradation of cell wall, membranes, cytoplasm and organelles were found.

- The anti-senescence effect of GA_3 on gentian petals at the ultrastructural level was observed. In the cells of the lower part of a petal around the vein region, appearance of senescence-associated features such as degradation of cell membranes, cytoplasm and organelles particularly chloroplasts including the thylakoids and chloroplast envelope was retarded. In the cells of the top part of a petal, more carotenoids-containing chromoplasts were found after GA_3 application.

- Both ethanol and STS can delay senescence of detached petunia petals. Treatment with 6% of ethanol or 0.3 mM of STS during the flower opening stage was particularly effective. Senescence parameters such as petal membrane damage, weight decline, ovary growth and ROS accumulation were all significantly suppressed particularly when detached petunia petals were treated with 6% ethanol.

- The anti-senescence effect of ethanol was confirmed in the isolated petunia petal system. The vase life of isolated petunia petals treated with 6% ethanol was about 2 times that of petals in the distilled water control. Membrane damage, weight decline, lower protein and total RNA levels were counteracted in ethanol-treated petals. In isolated petunia petals, senescence-associated formation and/or accumulation of ROS, particularly superoxide and hydrogen peroxide, was also inhibited or delayed by ethanol application. Activities of SOD, AP and POD were decreased during senescence but were promoted in response to ethanol treatment. The levels of ascorbate and photo-protective carotenoids in isolated petunia petals were not affected by ethanol treatment.

- By optimizing a range of critical PCR parameters such as primer combinations, cDNA concentrations and annealing temperatures, a reliable protocol has been

established for quantifying the expression level of Cu-Zn SOD gene using SYBR Green I based real-time RT-PCR.

- A 228 bp gene fragment of Cu-Zn SOD was isolated from petunia (var. 'hurrah') using RT-PCR.

- Down-regulation of Cu-Zn SOD mRNA level was observed during senescence of petunia petals.

- The anti-senescence effect of ethanol on petunia petals is partially related to the regulation of the antioxidative defense including promotion of Cu-Zn SOD gene expression. When compared to water-treated petals, there was about a 55-fold increase in Cu-Zn mRNA expression after 6 days of ethanol treatment.

- Down-regulation of GAPDH gene expression was observed during senescence of petunia petals.

- Ethanol not only prolonged vase life of petunia petals but also up-regulated GAPDH gene expression by a factor of about 16. The enhancement of general metabolic processes including promoting the key enzyme (GAPDH) in the control of glycolysis seems to be also involved in the regulation of petal senescence by ethanol treatment.

5.2 Basic aspects of flower development and senescence in this study

5.2.1 Importance of the present experimental designs

In the cut flower industry, cut gentian stems bearing flowers are commercially valuable. Therefore, studying cut gentian stems is of practical importance. However, it is difficult

to interpret the anti-senescence effect of post-harvest treatments on the flowers borne on the stems as there are various structures other than flowers which may interact with each other.

The single flower system is relatively better for revealing the direct effect of post-harvest treatment on vase life of gentian flowers. The challenge was on how to select flowers of exactly the same stage. This is difficult since detached gentian flowers do not open fully, and the visual difference in the blue color of gentian petals is only slight during senescence. The half petal system seems to be the best. The effect of GA_3 compared to that of water on a gentian petal was studied using this system.

In petunia, the anti-senescence effect of ethanol and STS was firstly confirmed in detached petunia flowers. Ethanol treatment plays a significant role in regulation of the sink-source relationship between petal and ovary. Retardation of petunia petal senescence by ethanol treatment might be related to inhibition of ovary growth. Furthermore, ethylene, a senescence mediator, is generally presumed to be produced and transferred from ovary to petal after flower pollination. The isolated petunia petal system used in this study is helpful in interpreting the results obtained using the detached petunia flower system. The anti-senescence effect of ethanol was confirmed again in the isolated petunia petal system, suggesting that nutrition deficiency due to ovary (strong growth sink) growth is not a central mechanism of manipulation by ethanol treatment of petunia petal vase life.

5.2.2 Sample selection strategy

Sample selection is also a very important and interesting consideration. When petunia petals become fully open (Stage OF), there is still a continuous growth period to follow. This growth period is called ‘beyond open flower’ (Stage BO) in this study. Although care was taken to harvest only flowers with uniform appearance from the same plant between these two stages, particularly those for pairwise comparison between the control

and a treatment, differences in the physiological age of the selected flowers might still exist. Differences in the range of vase life of flowers or petals between replicate experiments have been observed. These might be due to experimental imprecision in harvesting flowers of the required developmental stages.

5.2.3 Presentation of enzyme activity results

Initially, we tried to present enzyme activity data in three ways: based on per mg protein, per fresh weight and per petal. We found that during flower development, these three ways of presenting enzyme activity show similar trends. However, during flower senescence, a better way to present changes in petal enzyme activity is on the basis of per petal. This is because total fresh weight and protein content of a petal were continuously decreasing during senescence. Even on the basis of per petal variations could be evident due to the size difference of petals. Enzyme activity on the basis of the same original fresh weight of petal tissue would not have the error from petal size differences. It seems to be worthwhile to investigate this way to express and compare the levels of enzyme activities between different treatments during senescence in future studies.

Similarly, the staining intensities of isozyme bands on the gels are also related to enzyme activities of the isozyme bands. Equal fresh weight load in each well is valuable when enzyme activities were compared during petal development. However, equal petal tissue load in each well is better to show the changes of enzyme activity during senescence.

In this study, the results from isozyme analysis seem to be not always consistent with those from test-tube enzyme assays. For example, there were no significant differences in POD activities between ethanol- and water-treated petunia petals until day 10. However, the gel analysis indicated that there was a higher P_3 isozyme activity in the control petals when compared to that of ethanol-treated petals. The gel was run only under acidic conditions. In the future, it would be worthwhile to use isoeletric focusing gel electrophoresis to examine the full profiles of the antioxidative enzyme activities in crude petunia petal extracts.

5.3 Senescence mechanisms

5.3.1 Senescence and signal regulation

The molecular mechanisms involved in petal senescence are complex and still not fully understood. Signal transduction occupies a very important position in cellular processes including flower senescence. Signal molecules produced during flower senescence may bind to a cell surface or intercellular receptor and initiate the signal transudation pathway in which the regulation of protein phosphorylation, dephosphorylation and G-proteins are likely to be involved (Rubinstein, 2000). The generation of secondary massagers, such as calcium ions can greatly amplify the original signal and bring about the cellular response.

A common mechanism regulating petal senescence may involve free radicals, particularly the potential ROS signaling molecules (Behrend et al., 2003; Smirnoff, 2005). ROS, as signal molecules, play very important roles in plant biology in regulating growth, development and coordinating responses to abiotic and biotic stress. It has been found that about 113 genes are induced by ROS in plants (Desikan et al., 2001). Although superoxide and hydrogen peroxide have been considered to play key roles as ROS signal transduction molecules, recent studies have pointed to the existence of a ^{1}O2-specific signaling pathway (Roel et al., 2003). Moreover, another endogenous signaling free radical molecule, NO, produced in plants, is activated under abiotic and biotic stress. It interacts with ROS to trigger defense gene expression and cell death (Foissner et al., 2000). The protective effect of nitric oxide (NO) against senescence of rice leaves promoted by methyl jasmonate (MJ) is mediated through its ability to scavenge active oxygen species including H_2O_2 (Hung and Kao, 2004b).

The anti-senescence effect of ethanol on petunia petals is related to its regulation of reactive oxygen species, which might possibly be senescence-associated signals. Ethanol, as a hydroxyl radical scavenger, can interfere with the cellular oxidative / antioxidant status in petunia petals by inhibiting or delaying the formation and / or accumulation of

senescence-associated ROS molecules such as superoxide and H_2O_2. This changed oxidative / antioxidant status might affect a series of metabolic changes including changes in enzyme activity and gene expression. For example, the intensity of senescence-related isozyme P_3 was inhibited by ethanol application. High levels of both Cu-Zn SOD and GAPDH gene expression were observed following ethanol treatment.

In plants, ROS can be produced by enzymatic mechanisms such as NADPH oxidase and cell-wall-bound peroxidase (Vranova et al., 2002). Various other molecules such as calcium-binding proteins, protein kinase, and G protein would probably interact with NADPH oxidase and peroxidase to form macromolecular complexes. Together they could cooperate to regulate ROS production (Smirnoff, 2005). It was reported that ethanol treatment lowered general protein level in carnation petals (Podd et al., 2002a). Therefore the inhibition of those enzymes or proteins correlated with ROS production was possibly related to the anti-senescence effect of ethanol on restraining the rise in ROS levels. ROS can also be produced by non-enzymatic mechanisms such as electrons transferred to molecular oxygen during photosynthesis and respiration. Our results showed that chloroplast pigment levels were decreased in response to ethanol treatment. This suggests a decrease in general metabolic activity of chloroplasts. This reduced metabolic activity in chloroplasts could result in a low level of ROS production in ethanol-treated petunia petals.

In gentian, the effect of GA_3 on ROS is still not clear since both stimulation and inhibition of ROS generation have been reported. The ROS levels in gentian petals cannot be detected reliably by the method used in this study due to the dark color of petals. However, a decrease in ROS level in GA_3-treated leaves of *Pelargonium* cuttings has been reported (Rosenvasser et al., 2006). Further studies should require more sensitive and quantitative assays of superoxide and hydrogen peroxide for use in determination of these ROS levels in cut flower experiments.

There is increasing evidence showing that sugars can act as signals modulating many vital processes. Besides, they are widely accepted to play roles as providers of additional

respiration substrates and osmotic regulator for petal growth and development. For example, plants use hexokinase (HXK) as a glucose sensor to interrelate nutrient, light, and hormone signaling networks for controlling growth and development (Moore et al., 2003). In this study, 3% sucrose was found to be beneficial in delaying gentian flower senescence. Further studies are needed to examine more closely the mechanism involved.

Ethylene is another regulator involved in response of a plant to stresses and senescence (Grbic and Bleecker, 1995). Many external factors affect the senescence process possibly by the interaction of redox and hormones, particularly ethylene. Nutritional stress arising from nutrient deficiency can induce ethylene production through oxidative stress. This can be accompanied by a variety of morphological changes such as senescence and abscission (Lynch and Brown, 1997).

The effects of the anti-senescence treatments used in the present study are possibly related to their function in limiting the production of oxidative stress induced by a secondary signal such as ethylene. There are various reports showing the inhibitory effect of STS and ethanol on ethylene production and action (Podd et al., 2002a; Podd and Van, 1998; Zhang and Leung 2001). The almost complete inhibition of the activity of 1-aminocyclopropane-carboxylic acid oxidase and a decrease in the content of ACC (1-aminocyclopropane-1-carboxylic acid) was related to the anti-senescence effect of ethanol in carnation petals (Podd et al., 2002a).

Retardation of petal senescence in carnation by GA_3 is also thought to be mediated through ethylene regulation since ethylene production was also inhibited in the petal by the style and stigma (Saks and Van, 1992; Saks and Van, 1993b). The effect of a sugar on prolonging the vase life of cut flowers is related to a delay in ethylene biosynthesis. This could result from suppression of gene expression of ethylene forming enzymes, a decrease in the sensitivity to ethylene or inhibition of the transcriptional regulator in ethylene signalling (Pun and Ichimura, 2003; Yanagisawa et al., 2003; Zhang and Leung, 2001).

The potential cross-talks among different signal molecules such as sugar, ethylene and ROS are intimately related to petal senescence. It is well-known that external ethylene application enhances the senescence of ethylene-sensitive flowers (Bhattacharjee and Pal, 1999; Jiang et al., 1994). This ethylene-regulated petal senescence is associated with the increased synthesis of a specific protein (Jiang et al., 1994; Paliyath and Pinhero, 2000). This ethylene-induced protein in carnation petals was found to be a peroxidase (Jiang et al., 1999). Moreover, the production of ethylene from the conversion of ACC needs an oxidative environment. Ethylene has also been found to act as a positive regulator of ROS production (Overmyer et al., 2003).

The role of POD in senescence is complex. It has been reported that POD activity is concentrated in the vascular tissue of petals (Panavas and Rubinstein, 1998a). Several papers reported that hydrogen peroxide accumulated mainly in the vicinity of major and minor veins throughout the plant during oxidative stress (Moeder et al., 2002; Orozco-Cardenas and Ryan, 1999). It is well-known that H_2O_2 at low levels may act as a signal molecule to regulate metabolism. It can be speculated that the activity of H_2O_2-utilizing POD and ethylene-related genes may be influenced by hydrogen peroxide. On the other hand, the inhibitors of peroxidase (KCN and NaN_3) were reported to prevent ABA-induced H_2O_2 production in rice leaves. This suggests a role for peroxidase as a H_2O_2-generating enzymes for the formation / accumulation of H_2O_2 (Hung and Kao, 2004a). Thus a correlation between POD activity and hydrogen peroxide production is possible although their relationship may be complex.

During petunia petal senescence, P_3 was intensified in both the control and ethanol-treated petals (*Plate 4.14*), indicating the involvement and necessity of P_3 in the process of petal senescence. During closer observation of the staining of POD activity in samples shown in *Plate 4.14*, *C*, we found the isozymes of water-treated petals stained much quicker than those of ethanol-treated petals. Possibly this suggests a higher P_3 activity in water-treated petals although this is still semi-quantitative at best. It seems that the anti-senescence effect of ethanol might be associated with the inhibition of this senescence-

related POD isozyme activity. More complete understanding of the relationships among the isozyme $P_{3,}$ senescence, ethanol and ROS are required in further studies.

5.3.2 Senescence and antioxidants

Reducing the level of oxidative stress associated with aging by supplementing exogenous antioxidants might be an effective strategy (Finkel and Holbrook, 2000). Some success has been claimed regarding use of antioxidant therapy in mammalian models of disorders induced by oxidative stress. However, these strategies have had little or no effect in enhancing longevity (McCall and Frei, 1999; Yu, 1996).

Interestingly, similar strategies have been studied in plants. In *Iris*, several antioxidants such as benzoic acid, butylated hydroxytoluene, diphenylamine, propyl gallate, L-ascorbic acid and sodium benzoate had no effect on the time to tepal senescence (Celikel and Van, 1995). In addition, a range of other treatments which can increase free radical levels such as incubation with an oxidizing agent, inhibitors of antioxidants and elevated oxygen in the environment did not affect the time of visible tepal senescence (Bailly et al., 2001). However, petal senescence of cut carnations was delayed by a treatment with antioxidant sodium benzoate (Baker et al., 1977).

Although antioxidants do not always delay plant senescence, various factors such as plant growth regulators, influence senescence through modifying cellular oxidative / antioxidative metabolism. ABA and MJ can induce rice leaf senescence, which is associated with increased levels of hydrogen peroxide and MDA, decreased levels of reduced form of glutathione (GSH) and ascorbic acid (AsA), and an increase in the activities of antioxidative enzymes AP, SOD GR and CAT (Hung and Kao, 2004a; Hung and Kao, 2004b). On the contrary, in aleurone cells, ABA treatment greatly decreases the cellular sensitivity to H_2O_2-mediated programmed cell death (Gomez-Cadenas et al., 2001). In this study we found that GA_3 can delay gentian petal senescence in both detached flowers and those borne on cut stems. This was associated with an increase of anthocyanin and the activities of antioxidative enzymes (SOD and AP). This

improvement of antioxidant defense ability in response to GA_3 treatment would contribute to decreased oxidative stress / damage to gentian petal cells. However, this is different from the report on barley aleurone layers in which GA application repressed gene expression for antioxidative enzyme production and led to increased accumulation of ROS (Gomez-Cadenas et al., 2001). As a consequence, the aleurone cells became highly susceptible to oxidative damage and died earlier. In *Pelargonium* cuttings, GA_3 has been reported to restrain the rise in ROS level by an as yet unknown mechanism (Rosenvasser et al., 2006). In another study, GA was observed to stimulate the synthesis of ascorbate, which has also been reported as a signal molecule in regulating various metabolic activities (Puppo et al., 2005) in isolated *Arabidopsis* mitochondria (Millar et al., 2003).

Sucrose has an effect on delaying senescence of flowers borne on cut gentian stems. However, in preliminary observations (not shown here), the anti-senescence effect of sucrose could not be confirmed in the single gentian flower system. This suggests that the role of sucrose is at least partially related to its effect on the other parts of the cut stems such as leaves and stem tissues. Sucrose can serve as a respiration substrate and contribute to a balanced osmotic pressure in these tissues. It may also affect the cellular redox state through regulation of the antioxidative defense system since carbon skeletons are essential to the synthesis of numerous compounds involved in antioxidative defense (Couee et al., 2006). For example, glucose is the main initial precursor for synthesis of carotenoids and ascorbate (Smirnoff et al., 2001). It could provide carbon skeletons of amino acids, including Cys, Glu, and Gly which are the building blocks of glutathione (Noctor and Foyer, 1998). In tobacco, although there is no or only little difference in the activities of the antioxidative enzymes, glucose-fed leaves have an increased reductive capacity due to an increase in the contents of both glutathione and ascorbate (Polle and Eiblmeier, 1995). Similarly, in harvested broccoli florets, sucrose feeding can reverse the loss of ascorbate (Nishikawa et al., 2005).

In petunia, ethanol as a hydroxyl radical can delay petal senescence. The decreased ROS level in ethanol-treated petals is consistent with higher activities of SOD and AP as well

as enhanced gene expression of Cu-Zn SOD. Similarly, thiourea, another scavenger of hydroxyl radical, prevented H_2O_2-promoted senescence in detached rice leaves (Luijten et al., 1998). It was able to prevent a H_2O_2-induced decrease in the activities of both SOD and AP. A detailed time course of ROS production in responses to ethanol treatment in petunia petals is worthy of future investigations.

5.3.3 Senescence and protein degradation

Another important process contributing to flower senescence is protein degradation and remobilization which is mediated through the action of specific proteases and protein ubiquitination (Jones et al., 1995; Sugawara et al., 2002; Wagstaff et al., 2002).

In carnations, the protein content of ethanol-treated flowers was significantly lower than that of control flowers due to a general rather than specific loss of proteins. This could affect the metabolism of the flowers, preventing enzyme-mediated reactions as well as cell growth and development (Podd et al., 2002a). This seemed to be different with the present result as delaying petunia petal senescence by ethanol was associated with a significant retardation of a decrease in the petal protein content. Possibly, this might be due to different protein methods used or there might be some differences between different types of flowers.

The higher protein level in ethanol-treated petunia petals suggested possibly more protein synthesis and / or less protein degradation. It would be interesting to investigate if there is a decrease in protein degradation due to a decreased activity or expression of proteolytic enzymes. On the other hand, reduction in ROS levels after ethanol treatment probably led to limited oxidative stress and oxidative damage on enzymes, RNA and DNA. This could result in more proteins found in ethanol-treated petals.

Pigment degradation has been found to be dependent on the synthesis and transcription of new proteins, and modulated by protein synthesis and transcription inhibitors (Eason and De, 1995; Vaknin et al., 2005). It has been reported that the proteolytic degradation of

ribulose-1, 5-bisphosphate carboxylase / oxygenase, the induction of thiol-protease synthesis and the transcription of cysteine protease homolog PeSAG12-1 during senescence were suppressed in *Pisium sativum* and *Pelargonium* cuttings by GA during senescence (Aguero et al., 1996; Cercos and Carbonell, 1993; Cercos et al., 1992; Rosenvasser et al., 2006). The effect of GA_3 on pigment degradation and senescence of gentian petals can be investigated further in relation to these aspects of regulation of levels of the appropriate proteins.

Ubiquitin-mediated proteolysis, which is modulated by cellular redox state (Obin et al., 1998), is another critical regulatory mechanism controlling many biological processes including petal development and senescence. Upon oxidative stress, cells show an increase in the ratio of oxidized glutathione to reduced glutathione. This might covalently modify enzymes involved in the attachment of ubiquitin to substrate proteins since most of them contain sulfhydryls in their active site (Jahngen et al., 1997). SKP1, cullin / CDC53, F-box protein (SCF) complexes, possibly involved in gibberellin signaling in plants, play important roles in selecting specific substrates for proteolysis by facilitating the ligation of ubiquitin to specific proteins (Zhao et al., 2003). It remains to be determined if the anti-senescence effect of GA_3 on gentian petals is at least partially mediated through an ubiquitin-proteasome pathway.

5.4 Manipulation of cut flower senescence

It is very important to identify and elucidate the nature of the senescence-inducing signals originating within the plant body. Regarding the initiation of flower senescence, we need to have a more comprehensive understanding of this process. This is required before satisfactory strategies can be devised to modify the signal transduction process leading to more prolonged flower vase life.

Using classical conventional crossing and selection techniques, the vase life of cut flowers can be extended. For example, flowers with low ethylene production or low sensitivity to ethylene have been obtained using the traditional technology (Onozaki et

al., 2001). Genetic alterations including deletion, translocation, duplication, rearrangement and mutation of one or more senescence-associated genes are possible although achieving these might take a long time.

By means of genetic engineering to manipulate hormone contents or alter the responsiveness of a plant to hormones, plant growth regulators is very attractive. One of the various possible reasons for senescence is an internal hormonal imbalance (Ferrante et al., 2004). The ratio of 'inhibitor hormones' (ethylene, ABA and methyl jasmonate) and 'enhancer hormones' (cytokinin and GA) is possibly crucial to the regulation of growth or senescence process. A climacteric pattern in ethylene production and an increase in ethylene sensitivity were associated with senescence of ethylene sensitive flowers (Whitehead and Vasiljevic, 1993). Manipulation of an ethylene receptor gene has been found to delay flower senescence (Wang and Kumar, 2004). Overexpression of a cytokinin synthase gene delays petunia corolla senescence. At the same time this decreases or delays ethylene production, sensitivity to ethylene and an increase in ABA level (Chang et al., 2003). This suggests that various plant growth regulators may cooperate together to affect the process of flower senescence.

A common mechanism involved in delaying petal senescence of petunia by ethanol and gentian by GA_3 is modifying the cellular oxidative / antioxidant status including upregulation of antioxidant SOD activity in the petals. Since different SOD genes have been characterized and cloned in several plants (Gao et al., 2005; Lee et al., 1999; Temperton et al., 1996; Vyas and Kumar, 2005), they may be expressed in transgenic plants to provide interesting insights into their relative contributions to modulating senescence-associated oxidative stress. A study of senescence of cut flowers from a transgenic plant with altered expression of SOD genes is highly desirable. This resembles studies using cloned flower color genes to open up a wide range of applications, for example, creation of cultivars with a new flower color range (Wang and Peng, 2000). From a practical point of view, manipulation of genes coding for antioxidative enzymes such as Cu-Zn SOD, could be beneficial to the longevity extension of cut flowers in the future.

5.5 Conclusions

A central problem in plant developmental biology is concerned with the regulation of plant senescence. Investigations into this problem, particularly flower senescence, are of great interests to the cut flower industry as well. In this project, we mainly studied the effect of GA_3 on the senescence of flowers borne on cut gentian stems, detached flowers and isolated petals of gentian. In petunia, the effect of ethanol on senescence of detached flowers and isolated petals were studied. Changes at the morphological, biochemical, ultrastructural and molecular levels in response to these chemical treatments, were studied using some of these plant materials. From the results obtained in this study it can be concluded that:

- Although ethanol and GA_3 are not ideal as practical post-harvest treatments in the cut flower industry, they are useful in studies aiming to obtain a better understanding of regulation of cut flower senescence.

- A common mechanism involved in the anti-senescence effect of GA_3 on gentian petals and ethanol on petunia petals is at least partially mediated via modifying the cellular ROS / antioxidant status to cope with the senescence-associated oxidative stress in the petals.

- Regulation of pigment metabolism does not appear to a central mechanism in petal senescence since the anticipated significant increase in the level of antioxidative pigments, particularly carotenoid or anthocyanin, was not always observed following a post-harvest treatment. This might require further investigations.

5.6 Recommendations for future research

Arising from the present study, there are many points of immediate interests that could be investigated further. They are as follow:

- The effect of $GA_{3,}$ if any, on the leaves and stem tissues of cut gentian flower stems should be studied. This might yield better understanding of how GA_3 delays senescence of flowers borne on cut gentian stems.

- The peroxidase isozyme P_3 was more active during petunia petal senescence and the anti-senescence effect of ethanol was related to suppression of this P_3 activity. Further studies can include an assessment of its relationship to ROS production during petal senescence.

- It is hypothesized that during cut flower senescence, there will be an increase in oxidatively-damaged proteins and their removal is likely associated with an elevated activity in the ubiquitin-mediated protein degradation pathway. It is not known whether a decreased level of ROS, protein ubiquitination and the action of a specific protease are involved in the responses of gentian petals to GA_3 treatment. It is also not known whether a decrease in protein degradation is involved in the response of petunia petals following ethanol application. These require further investigations.

- Transfer of the gene fragment isolated from this study or complete cDNA of Cu-Zn SOD to generate transgenic plants. This will allow more in-depth studies into the relationship between this enzyme and potential tolerance to senescence-associated oxidative stress in petunia petals.

- Regulation of gene expression for antioxidative enzymes in gentian petals by GA_3 has not been studied before. This seems to be an interesting topic for further studies.

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Appendices

Appendix A: Solutions for gel electrophoresis

1. Preparation of separating gel and stacking gel for SDS-PAGE

Solution for Tris / Glycine (SDS-PAGE)	15% Separating gel (ml)	4% Stacking gel (ml)
dH_2O	3.000	7.500
1M Tris-HCl (pH 8.8)	11.250	0.000
1M Tris-HCl (pH 6.8)	0.000	1.250
30% Acrylamide : Bis	15.000	1.000
10% SDS	0.300	0.100
10% Ammonium persulphate	0.300	0.040
TEMED	0.015	0.015

2. Sample buffer (5×) for SDS-PAGE

Stock solution	10 ml
1M Tris-HCl (pH 6.8)	0.600
50% Glycerol	5.000
10% SDS	2.000
ß- mercaptoethanol	0.500
1% Bromophenol blue	1.000
dH_2O	0.900

3. Electrophoresis buffer for SDS-PAGE

Electrophoresis buffer	1 Litre
Tris Base	4.540 g
Glycine	21.600 g
SDS	1.500 g

4. Commassie blue gel staining

Staining solution	1 Litre
Commassie Blue R-250	1.00 g
Methanol	500 ml
Glacial acetic acid	100 ml
dH_2O	400 ml

5. Commassie blue gel destaining

Destaining solution	1 Liter
Methanol	50.0 ml
Glacial acetic acid	100 ml
dH_2O	850 ml

Notes: Gel preparation and gel electrophoresis running buffer under non-denaturing conditions are the same as SDS-PAGE, except that SDS was omitted from non-denaturing gels. Samples were mixed with 60% sucrose (2:1) before loading samples on the wells. All stock solutions stores at 4°C.

Appendix B: Procedures used for molecular work

1. DNA sequences of specific PCR primers for interested genes

Genes	Reference gene bank ACC. no	Reference gene source	Specific primer pair sequences
Cu-Zn SOD	X14352	Petunia x hybrida	F1: 5'AATCTTCACCACCACAAGCACTA 3' F2: 5' ATTGCTTCCTCTAACACCAACTCT 3' R1: 5' CAACACCCTCAACATTGGAAGT 3' R2: 5' TAAGAGTGACAACACCCTCAACAT 3'
GAPDH	X60346	Petunia x hybrida	F1: 5' TAAGGCTGTTGGAAAGGTGCTA 3' F2: 5' GGAGAAAGAAGCCACCTATGA 3' R1: 5' TGTTGTCCCCGATAAAGTCAGTA 3' R2: 5' TGTAGCCCATTTCGTTGTCGTA 3'
Actin	AY038063	Petunia x hybrida	F: 5' GAGCTATGAGTTACCTGATGGACA R: 5' GTAATCTCCTTGCTCATCCTATCA
18S rRNA	AJ236020	Petunia axillaris	F: 5' TACCGTCCTAGTCTCAACCATAA 3' R: 5' AGAACATCTAAGGGCATCACA 3'

2. Procedure for pre-treatment of RNA by DNase I

Conditions	Components
Reaction mixture	RNA 10 µg 1-fold DNase I buffer to 100 µl DNase I (1 unit)
Incubation	37°C for 10 min
Protecting RNA from degradation	by 1 µl 0.5 mM EDTA added
Denaturing DNase	75 °C for 10 min
Precipitate RNA	in 500 µl isopropernol for standing at room temperature for 10 min and then 14000 rpm 12 min at 4°C
Wash RNA	in 1 ml 75% ethanol and then 7500 rpm for 7 min at 4 °C
Dissolve RNA	in DEPC-water with 1-fold RNAsecure (10 µl) and then 65 °C for 10 min to activate its ability to protect RNA

3. Reverse transcription procedure

Condition	Components	Volume (µl)	Final concentrations
Denaturation of RNA second structure:	RNA	X µl	1 µg
	Oligo-p$(dT)_{15}$ primer	1 µl	50 pmol
	Random primer p$(dN)_6$	1 µl	1.6 µg
	DEPC-water	13-1-1-X	variable
	65°C for 10 min Immediately on ice for 5 min		
Master mix preparation:	5 fold RT buffer	4 µl	1-fold
	RNAsin (40 units/µl)	0.5 µl	20 units
	dNTPs (10 mM)	2 µl	1 mM each
	Transcriptor Reverse Transcriptase	0.5 µl	10 U
	Total	20 µl	
Incubation:	25°C for 10 min first Then 55 °C for 30 min		
Denaturing (RT enzyme)	85 °C for 5 min first Then place on ice		

4. Program setting for amplification of Cu-Zn SOD, GAPDH, 18S rRNA and actin genes by real-time PCR

Program setting	Target temperature (°C)	Reaction time (s)	Temperature transition (°C/s)
Activation (1 cycle)	95	10.0 min	20
PCR (40-45 cycles)			
Denaturing	95	30 s	20
Annealing	55	60 s	20
Elongation	72	30 s	20
Fluorescence acquisition	74	10 s	20
Melting (1 cycle)			
Segment 1	95	60 s	20
Segment 2	55	30 s	20
Segment 3	95	30 s	0.1
Fluorescence acquisition			

5. Real-time PCR reaction mixtures for the amplification of cDNAs of Cu-Zn SOD, GAPDH, 18S rRNA and actin in petunia petals

Reaction mixture	Components	Volume (µl)	Final concentration in reaction mixture
Template	cDNA	1.0 µl	50-fold dilution
	Former primer (18 µM)	1.0 µl	900 nM
	Reverse primer (18 µM)	1.0 µl	900 nM
Master mix:	SYBR Green I	10.0 µl	1x
	DEPC-water	7.0 µl	
Total		20.0 µl	